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(54) PROTEIN EXPRESSION SYSTEMS

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(57) **ABSTRACT**

The present invention provides an improved expression system for the production of recombinant polypeptides utilizing auxotrophic selectable markers. In addition, the present invention provides improved recombinant protein production in host cells through the improved regulation of expression.

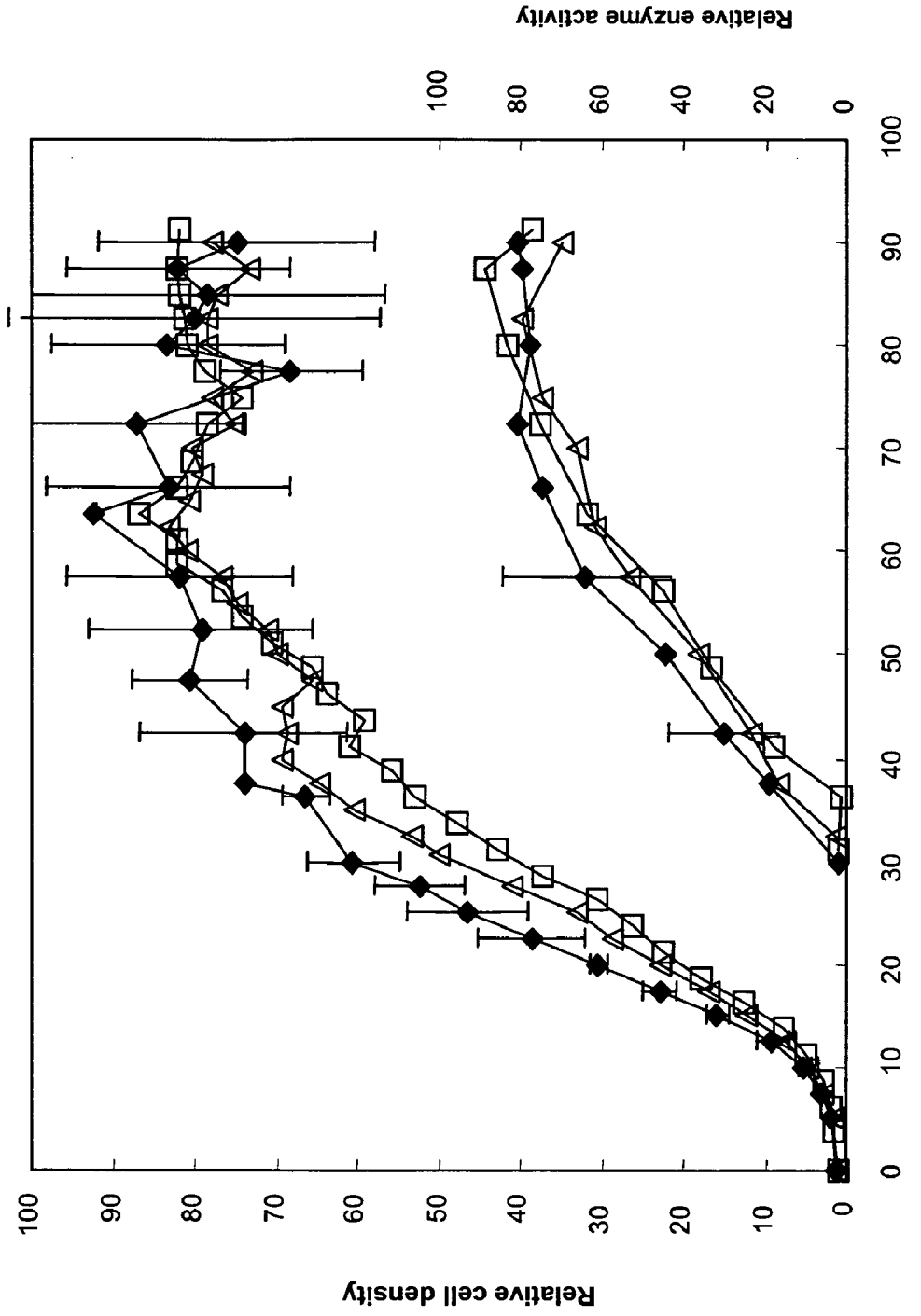
Relative cell density
Relative enzyme activity
Relative fermentation time
0
10
20
30
40
50
60
70
80
90
100

Figure 1

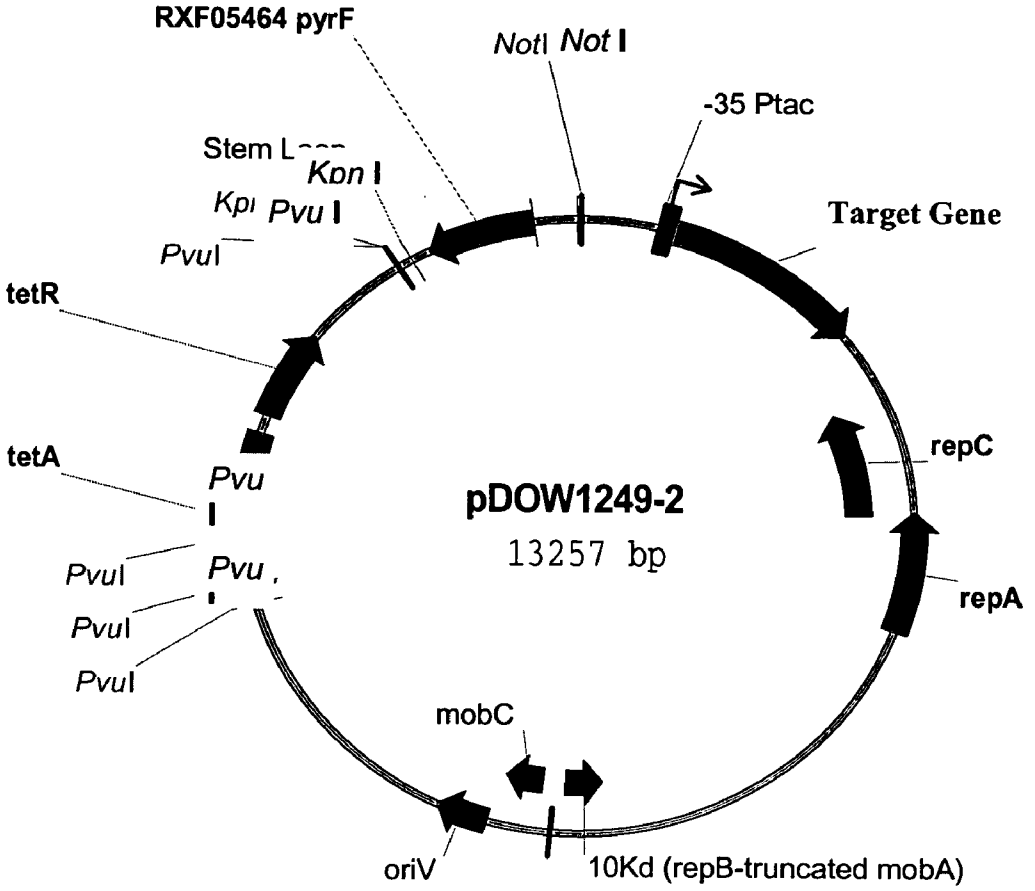
RXF05464 pyrF
NotI Not I
-35 Ptac
Stem L
Kpn I
Kpi Pvu I
PvuI
Target Gene
tetR
tetA
Pvu I
repC
pDOW1249-2
13257 bp
PvuI
Pvu I
repA
PvuI
PvuI
mobC
oriV
10Kd (repB-truncated mobA)

Figure 2

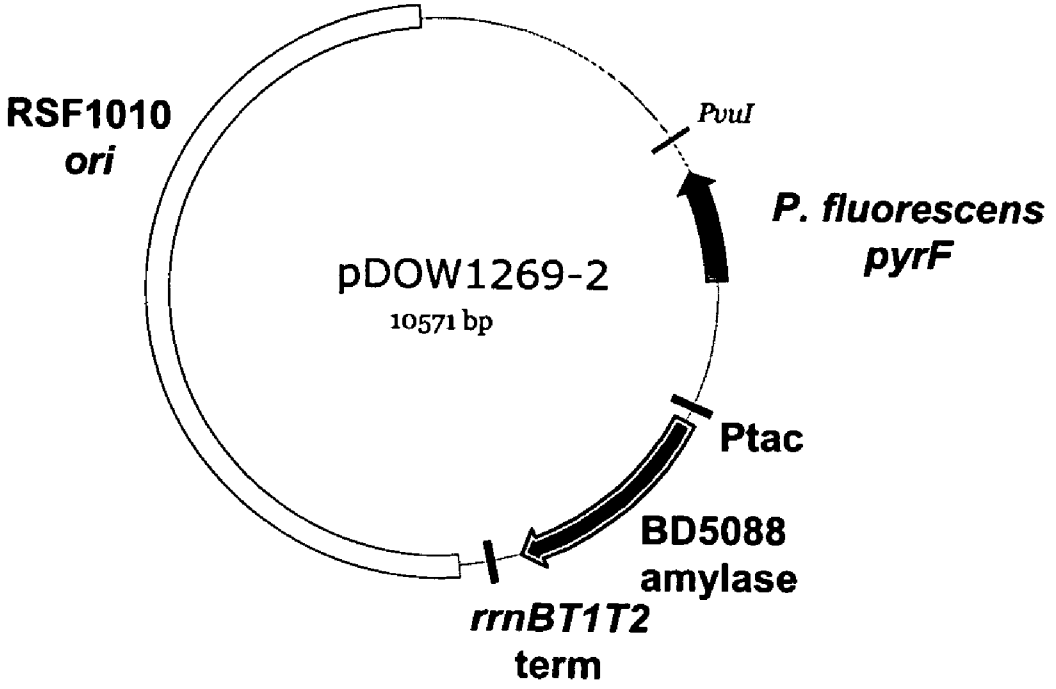
RSF1010
ori
PvuI
P. fluorescens
pyrF
pDOW1269-2
10571 bp
Ptac
BD5088
amylase
rrnBT1T2
term

Figure 3

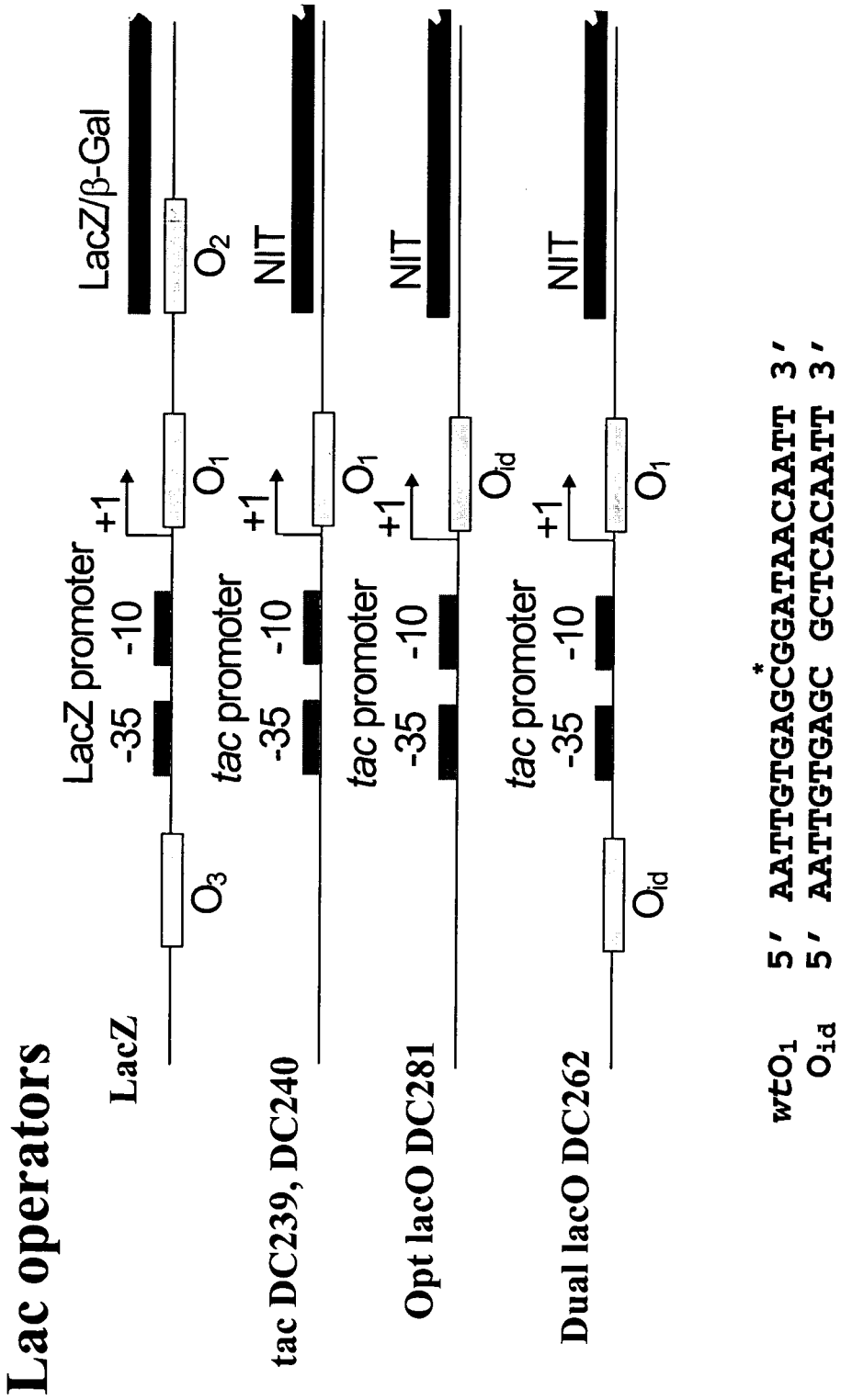
Lac operators
LacZ
LacZ promoter
-35
-10
+1
O3
O1
O2
LacZ/β-Gal
tac DC239, DC240
tac promoter
-35
-10
+1
O1
NIT
Opt lacO DC281
tac promoter
-35
-10
+1
Oid
NIT
Dual lacO DC262
tac promoter
-35
-10
+1
Oid
O1
NIT
wtO1 5′ AATTGTGAGCGGATAACAATT 3′
Oid 5′ AATTGTGAGC GCTCACAATT 3′

Figure 4

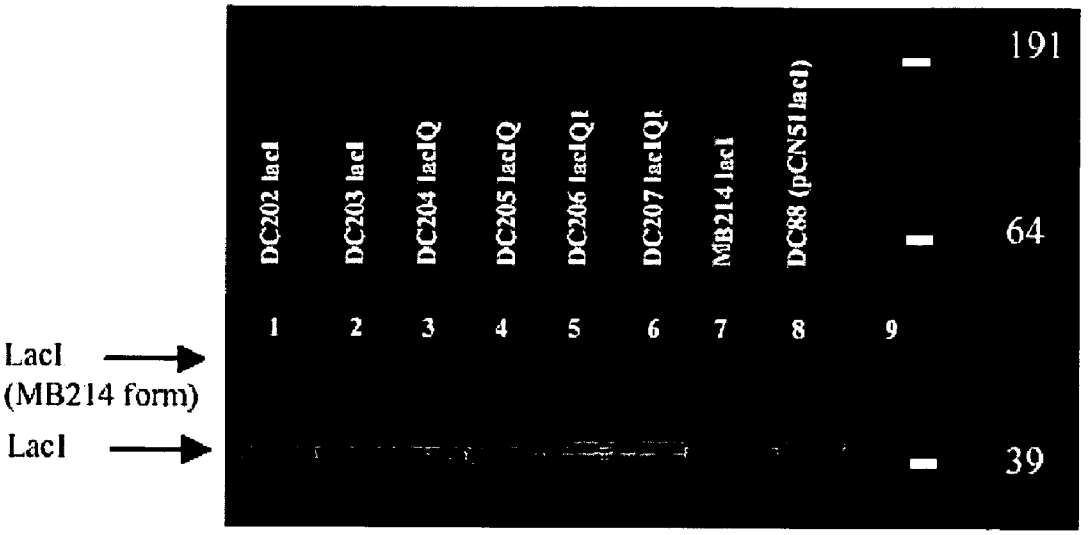
DC202 lacI
DC203 lacI
DC204 lacIQ
DC205 lacIQ
DC206 lacIQ1
DC207 lacIQ1
MB214 lacI
DC88 (pCN51 lacI)
1
2
3
4
5
6
7
8
9
191
64
39
LacI
(MB214 form)
LacI

Figure 5

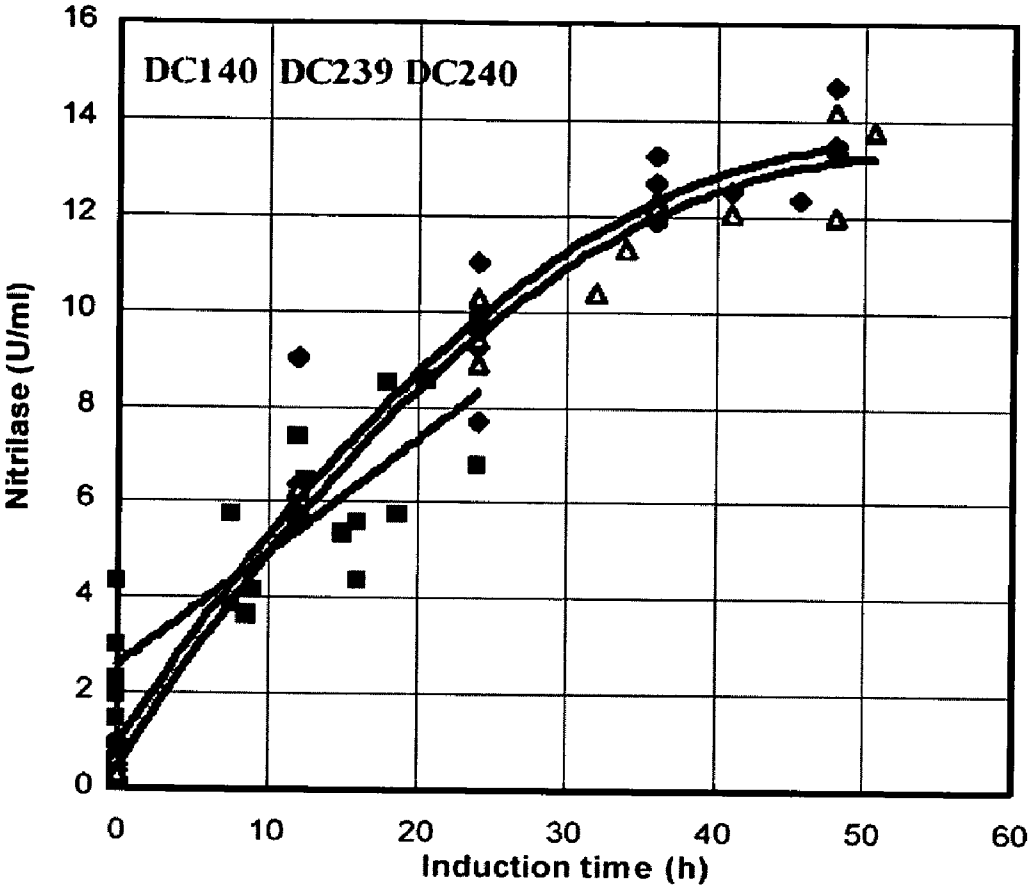
DC140 DC239 DC240
Nitrilase (U/ml)
Induction time (h)
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60

Figure 6

PROTEIN EXPRESSION SYSTEMS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional patent application Ser. No. 60/523,420 filed Nov. 19, 2003, entitled "Improved *Pseudomonas* Expression Systems with Auxotrophic Selection Markers," and U.S. Provisional patent application 60/537,147 filed Jan. 16, 2004, and entitled "Bacterial Expression Systems with Improved Repression."

FIELD OF THE INVENTION

[0002] The present invention provides an improved expression system for the production of recombinant polypeptides utilizing auxotrophic selectable markers. In addition, the present invention provides improved recombinant protein production in host cells through the improved regulation of expression.

BACKGROUND OF THE INVENTION

[0003] The use of bacterial cells to produce protein based therapeutics is increasing in commercial importance. One of the goals in developing a bacterial expression system is the production of high quality target polypeptides quickly, efficiently, and abundantly. An ideal host cell for such an expression system would be able to efficiently utilize a carbon source for the production of a target polypeptide, quickly grow to high cell densities in a fermentation reaction, express the target polypeptide only when induced, and grow on a medium that is devoid of regulatory and environmental concerns.

[0004] There are many hurdles to the creation of a superior host cell. First, in order to produce a recombinant polypeptide, an expression vector encoding the target protein must be inserted into the host cell. Many bacteria are capable of reverting back into an untransformed state, wherein the expression vector is eliminated from the host. Such revertants can decrease the fermentation efficiency of the production of the desired recombinant polypeptide.

[0005] Expression vectors encoding a target peptide typically include a selection marker in the vector. Often, the selection marker is a gene whose product is required for survival during the fermentation process. Host cells lacking the selection marker, such as revertants, are unable to survive. The use of selection markers during the fermentation process is intended to ensure that only bacteria containing the expression vector survive, eliminating competition between the revertants and transformants and reducing the efficiency of fermentation.

[0006] The most commonly used selection markers are antibiotic resistance genes. Host cells are grown in a medium supplemented with an antibiotic capable of being degraded by the selected antibiotic resistance gene product. Cells that do not contain the expression vector with the antibiotic resistance gene are killed by the antibiotic. Typical antibiotic resistance genes include tetracycline, neomycin, kanamycin, and ampicillin. The presence of antibiotic resistance genes in a bacterial host cell, however, presents environmental, regulatory, and commercial problems. For example, antibiotic resistance gene-containing products (and products produced by the use of antibiotic resistance gene) have been identified as potential biosafety risks for environmental, human, and animal health. For example, see M. Droge et al., Horizontal Gene Transfer as a Biosafety issue: A natural phenomenon of public concern, J. Biotechnology. 64(1): 75-90 (17 Sept. 1998); Gallagher, D. M., and D. P. Sinn. 1983. Penicillin-induced anaphylaxis in a patient under hypotensive anaesthesia. *Oral Surg. Oral Med. Oral Pathol.* 56:361-364; Jorro, G., C. Morales, J. V. Braso, and A. Pelaez. 1996. Anaphylaxis to erythromycin. Ann. *Allergy Asthma Immunol.* 77:456-458; F. Gebhard & K. Smalla, Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA, Appl. & Environ. Microbiol. 64(4):1550-54 (Apr. 1998); T. Hoffinann et al., Foreign DNA sequences are received by a wild type strain of *Aspergillus niger* after co-culture with transgenic higher plants, Curr. Genet. 27(1): 70-76 (Dec. 1994); DK Mercer et al., Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonoii* DL1 by plasmid DNA in human saliva, Appl. & Environ. Microbiol. 65(1):6-10 (Jan 1999); R. Schubbert et al., Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA, PNAS USA 94:961-66 (Feb. 4, 1997); and AA Salyers, Gene transfer in the mammalian intestinal tract, Curr. Opin. in Biotechnol. 4(3):294-98 (Jun. 1993).

[0007] As a result of these concerns, many governmental food, drug, health, and environmental regulatory agencies, as well as many end users, require that antibiotic resistance gene nucleic acid be removed from products or be absent from organisms for use in commerce. In addition, evidence demonstrating clearance of the selection antibiotics from the final product must be provided in order to secure regulatory clearance. The United Kingdom, Canada, France, the European Community, and the United States have all addressed the use of antibiotic resistance genes in foods, animal feeds, drugs and drug production, including recombinant drug production. Clearance of these agents, and especially demonstrating such clearance, is expensive, time consuming, and often only minimally effective.

[0008] Because of the concerns inherent in the use of antibiotic resistance genes for selection in the production of recombinant polypeptides, alternative selection methods have been examined.

[0009] Auxotrophic Selection Markers

[0010] Auxotrophic selection markers have been utilized as an alternative to antibiotic selection in some systems. For example, auxotrophic markers have been widely utilized in yeast, due largely to the inefficiency of antibiotic resistance selection markers in these host cells. See, for example, JT Pronk, (2002) "Auxotrophic yeast strains in fundamental and applied research," App. & Envirn. Micro. 68(5): 2095-2100; Boeke et al., (1984) "A positive selection for mutants lacking orotodine-5'-phosphate decarboxylase activity in yeast; 5-fluoro-orotic acid resistance," Mol. Gen. Genet. 197: 345-346; Botstein & Davis, (1982) "Principles and practice of recombinant DNA research with yeast," p.607-636, in J N Strathern, E W Jones. And JR Broach (ed.), The molecular biology of the yeast Saccharomyces cerevisiae, Metabolism and gene expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; Cost & Boeke, (1996) "A useful colony color phenotype associated with the

yeast selectable/counter selectable marker MET15," Yeast 12: 939-941. However, yeast expression systems due not provide the potential speed and efficiency for producing target proteins that bacterial systems do.

[0011] Auxotrophic marker selection in bacteria has also previously been described. See, for example, U.S. Pat. Nos. 4,920,048, 5,691,185, 6,291,245, 6,413,768, 6,752,994, Struhl et al. (1976) PNAS USA 73; 1471-1475;; MacCormick, C. A., et al., (1995) "Construction of a food-grade host/vector system for *Lactococcus lactis* based on the lactose operon,"*FEMS Microbiol. Lett.* 127:105-109; Dickely et al. (1995), "Isolation of *Lactococcus lactis* nonsense suppressors and construction of a food-grade cloning vector,"*Mol. Microbiol.* 15:839-847; Sørensen et al., (2000) "A food-grade cloning system for industrial strains of *Lactococcus lactis," Appl. Environ. Microbiol* 66:1253-1258; Fiedler & Skerra, (2001) "proBA complementation of an auxotrophic E.coli strain improves plasmid stability and expression yield during fermenter production of a recombinant antibody fragment," Gene 274: 111-118.

[0012] The use of auxotrophic selection markers in the previously described commercial scale bacterial fermentation systems has drawbacks that limit their use. A major drawback, as noted in U.S. Pat. No. 6,413,768, is that nutritional auxotrophic selection marker systems generally suffer from cross feeding. The term cross feeding refers to the ability of a first cell, auxotrophic for a particular metabolite, to survive in the absence of the metabolite by obtaining its supply of that metabolite from its environment, and typically, from the medium for which the cell is auxotrophic by utilizing excreted intermediates of the metabolite, the metabolite itself, or a prototrophic enabling molecule produced by a second cell, prototrophic for the metabolite absent from the medium. See also G R Barker et al., Biochem. J. 157(1):221-27 (1976) (cross feeding of thymine in E.coli): T J Kerr & G J Tritz, J. Bact. 115(3):982-86 (Sep. 1973) (cross feeding of NAD in E.coli auxotrophic for NAD synthesis); G A Sprenger et al., FEMS Microbiol. Lett. 37(3):299-304 (1986) (selection of nalidixic acid to avoid the cross feeding problem).

[0013] Because cross feeding allows revertant bacteria to survive, cross feeding decreases the overall capacity of the fermentation process to produce the desired product at efficient and maximized levels due to the presence of fewer target protein producing host cells.

[0014] Expression Vector Control

[0015] Another hurdle to the creation of the ideal host cell is the inefficient and low level production of target polypeptides in the fermentation process. Controlling expression of the target protein until optimal host cell densities and fermentation conditions are reached allows for a more efficient and larger yield of polypeptide. The reasons for this are several fold, including a more efficient utilization of a particular carbon source and the reduction of extended metabolic stresses on the host cell.

[0016] In many cases, however, repression of expression of the target protein during cell growth can be imperfect, resulting in a significant amount of expression prior to the particular induction phase. This "leaky" repression results in host cell stress, inefficient utilization of carbon source due to metabolic energy being diverted from normal cell growth to transgene, and a delay in reaching optimal cell density induction points, resulting in a more lengthy and costly fermentation run, and often, a reduced yield of the target protein.

[0017] Therefore, it is an object of the present invention to provide an improved expression system for the production of target proteins, wherein the production is efficient, regulatable, and performed in a medium that minimizes of regulatory and environmental concerns.

[0018] It is another object of the present invention to provide organisms for use as host cells in an improved expression system for the production of target proteins.

[0019] It is still another object of the present invention to provide processes for the improved production of target proteins.

[0020] It is yet another object of the present invention to provide novel constructs and nucleic acids for use in an improved expression system for the production of target proteins.

SUMMARY OF THE INVENTION

[0021] It has been discovered that bacterial protein production can be improved by selecting as a host cell a Pseudomonad organism that is capable of non-antibiotic resistant, auxotrophic selection, and/or contains a chromosomal insert of a lacI gene or derivative.

[0022] Specifically, it has been discovered that the Pseudomonad organism *Pseudomonas fluorescens* is particularly well suited for this purpose. To this end, it has been surprisingly discovered that *Pseudomonas fluorescens* does not exhibit adverse cross feeding inhibition under auxotrophic selection during the high-cell density fermentation of recombinant polypeptides. Such a discovery allows for the use of auxotrophic *Pseudomonas fluorescens* as host cells in the efficient production of high levels of recombinant polypeptides, overcoming the drawbacks inherent with the use of antibiotic resistance selection markers and the problems of auxotrophic cross feeding present in other bacterial expression systems.

[0023] It has also been surprisingly discovered that the use of a LacI-encoding gene other than as part of a whole or truncated Plac-lacI-lacZYA operon in Pseudomonads surprisingly resulted in substantially improved repression of pre-induction recombinant protein expression, higher cell densities in commercial-scale fermentation, and higher yields of the desired product in comparison with previously taught lacI-lacZYA Pseudomonad chromosomal insertion (U.S. Pat. No. 5,169,760). This lacI insertion is as effective in repressing Plac-Ptac family promoter-controlled transgenes as a multi-copy plasmid encoding a LacI repressor protein in *Pseudomonas fluorescens,* thereby eliminating the need to maintain a separate plasmid encoding a LacI repressor protein in the cell and reducing potential production inefficiencies caused by such maintenance.

[0024] It has also been discovery that the use of dual lac operator sequences provides superior repression of recombinant protein expression prior to induction without a concomitant reduction in subsequent induction yields in *Pseudomonas fluorescens*

[0025] Therefore, in one aspect of the present invention, Pseudomonad organisms are provided for use as host cells in the improved production of proteins.

[0026] In one embodiment, the Pseudomonad organisms have been genetically modified to induce an auxotrophy. In a particular embodiment, the Pseudomonad organism is *Pseudomonas fluorescens*. In one embodiment, the auxotrophy is a result of genetic modifications to at least one nitrogenous base compound biosynthesis gene, or at least one amino acid biosynthesis gene. In a further embodiment, the genetic modification is to a gene encoding an enzyme active in the uracil biosynthetic pathway, the thymidine biosynthetic pathway, or the proline biosynthetic pathway. In still a further embodiment, the genetic modification is to the pyrF gene encoding orotidine-5'-phosphate decarboxylase, the thyA gene encoding thymidylate synthase, or the proC gene encoding Δ^1-pyrroline-5-carboxylate reductase.

[0027] In another embodiment, the present invention provides Pseudomonad organisms that have been genetically modified to provide at least one copy of a LacI-encoding gene inserted into the genome, other than as part of the whole or truncated Plac-lacI-lacZYA operon. In a particular embodiment, the Pseudomonad host cell is *Pseudomonas fluorescens*. In one embodiment, the Pseudomonad contains a native *E.coli* lacI gene encoding the LacI repressor protein. In another embodiment, the Pseudomonad cell contains the $lacI^Q$ gene. In still another embodiment, the Pseudomonad cell contains the $lacI^{Q1}$ gene.

[0028] In another embodiment, a Pseudomonad organism is provided comprising a nucleic acid construct containing a nucleic acid comprising at least one lacO sequence involved in the repression of transgene expression. In a particular embodiment, the Pseudomonad host cell is *Pseudomonas fluorescens*. In one embodiment, the nucleic acid construct comprises more than one lacO sequence. In another embodiment, the nucleic acid construct comprises at least one, and preferably more than one, lacOid sequence. In one embodiment, the nucleic acid construct comprises a lacO sequence, or derivative thereof, located 3' of a Plac family promoter, and a lacO sequence, or derivative thereof, located 5' of a Plac family promoter. In a particular embodiment, the lacO derivative is a lacOid sequence.

[0029] In a further embodiment, the present invention provides Pseudomonad organisms that have been genetically modified to induce an auxotrophy and further modified to contain a chromosomal insertion of a native *E.coli* lacI gene, $lacI^Q$ gene, or $lacI^{Q1}$ gene other than as part of a whole or truncated Plac-lacI-lacZYA operon. In another embodiment, the Pseudomonad organism is further modified to contain a nucleic acid construct comprising at least one lacO sequence involved in the repression of transgene expression. In a particular embodiment, the Pseudomonad organism is a *Pseudomonas fluorescens*.

[0030] In another aspect of the present invention, nucleic acid sequences are provided for use in the improved production of proteins.

[0031] In one embodiment, nucleic acid sequences encoding prototrophy-restoring enzymes for use in an auxotrophic Pseudomonad host cells are provided. In a particular embodiment, nucleic acid sequences encoding nitrogenous base compound biosynthesis enzymes purified from the organism *Pseudomonas fluorescens* are provided. In one embodiment, nucleic acid sequences encoding the pyrF gene in *Pseudomonas fluorescens* is provided (SEQ. ID No.s 1 and 3). In another embodiment, a nucleic acid sequence encoding the thyA gene in *Pseudomonas fluorescens* is provided (SEQ. ID. No. 4). In still another embodiment, nucleic acid sequences encoding an amino acid biosynthetic compound purified from the organism *Pseudomonas fluorescens* are provided. In a particular embodiment, a nucleic acid sequence encoding the proC gene in *Pseudomonas fluorescens* is provided (SEQ. ID No.s 6 and 8).

[0032] In another aspect, the present invention produces novel amino acid sequences which are the products of the novel nucleic acid expression.

[0033] In still another aspect of the present invention, nucleic acid constructs are provided for use in the improved production of peptides.

[0034] In one embodiment, a nucleic acid construct for use in transforming a Pseudomonad host cell comprising a) a nucleic acid sequence encoding a recombinant polypeptide, and b) a nucleic acid sequence encoding a prototrophy-enabling enzyme is provided. In another embodiment, the nucleic acid construct further comprises c) a Plac-Ptac family promoter. In still another embodiment, the nucleic acid construct further comprises d) at least one lacO sequence, or derivative, 3' of a lac or tac family promoter. In yet another embodiment, the nucleic acid construct further comprises e) at least one lacO sequence, or derivative, 5' of a lac or tac family promoter. In one embodiment, the derivative lacO sequence can be a lacOid sequence. In a particular embodiment, the Pseudomonad organism is *Pseudomonas fluorescens*.

[0035] In one embodiment of the present invention, nucleic acid constructs are provided for use as expression vectors in Pseudomonad organisms comprising a) a nucleic acid sequence encoding a recombinant polypeptide, b) a Plac-Ptac family promoter, c) at least one lacO sequence, or derivative, 3' of a lac or tac family promoter, d) at least one lacO sequence, or derivative, 5' of a lac or tac family promoter. In one embodiment, the derivative lacO sequence can be a lacOid sequence. In one embodiment, the nucleic acid construct further comprises e) a prototrophy-enabling selection marker for use in an auxotrophic Pseudomonad cell. In a particular embodiment, the Pseudomonad organism is *Pseudomonas fluorescens*.

[0036] In another aspect of the present invention, modified cells are provided for use in the improved production of proteins.

[0037] In one embodiment, an auxotrophic Pseudomonad cell is provided that has a nucleic acid construct comprising i) a recombinant polypeptide, and ii) a prototrophy-enabling nucleic acid. In another embodiment, the nucleic acid construct further comprises iii) a Plac-Ptac family promoter. In still another embodiment, the nucleic acid construct further comprises iv) more than one lacO sequence. In one embodiment, the Pseudomonad is an auxotrophic *Pseudomonas fluorescens* cell. In a further embodiment, the invention further comprises auxotrophic Pseudomonad organisms, including *Pseudomonas fluorescens,* that have been further genetically modified to contain a chromosomal insertion of a native *E.coli* lacI gene, $lacI^Q$ gene, or $lacI^{Q1}$ gene other than as part of a whole or truncated Plac-lacI-lacZYA operon.

[0038] In another embodiment, a Pseudomonad cell is provided that comprises a lacI transgene, or derivative thereof, other than as part of a whole or truncated Plac-lacI-lacZYA operon, inserted into the chromosome, and b) a nucleic acid construct comprising i) a recombinant polypeptide, and ii) a Plac-Ptac family promoter. In still another embodiment, the nucleic acid construct further comprises iii) at least one lacO sequence, and preferably, more than one lacO sequence. In one embodiment, the lacO sequence is a lacOid sequence. In one embodiment, the Pseudomonad has been further modified to induce auxotrophy. In one embodiment, the Pseudomonad cell is a *Pseudomonas fluorescens*.

[0039] In one aspect of the present invention, processes of expressing recombinant polypeptides for use in improved protein production are provided.

[0040] In one embodiment, the process provides expression of a nucleic acid construct comprising nucleic acids encoding a) a recombinant polypeptide, and b) a prototrophy-restoring enzyme in a Pseudomonad that is auxotrophic for at least one metabolite. In an alternative embodiment, the Pseudomonad is auxotrophic for more than one metabolite. In one embodiment, the Pseudomonad is a *Pseudomonas fluorescens* cell. In a particular embodiment, a recombinant polypeptide is expressed in a Pseudomonad that is auxotrophic for a metabolite, or combination of metabolites, selected from the group consisting of a nitrogenous base compound and an amino acid. In a more particular embodiment, recombinant polypeptides are expressed in a Pseudomonad that is auxotrophic for a metabolite selected from the group consisting of uracil, proline, and thymidine. In another embodiment, the auxotrophy can be generated by the knock-out of the host pyrF, proC, or thyA gene, respectively. An alternative embodiment, recombinant polypeptides are expressed in an auxotrophic Pseudomonad cell that has been genetically modified through the insertion of a native *E.coli* lacI gene, $lacI^Q$ gene, or $lacI^{Q1}$ gene, other than as part of the PlacI-lacI-lacZYA operon, into the host cell's chromosome. In one particular embodiment, the vector containing the recombinant polypeptide expressed in the auxotroph comprises at least one lacOid operator sequences. In one particular embodiment, the vector containing the recombinant polypeptide expressed in the auxotrophic host cell comprises at least two lac operator sequences, or derivatives thereof. In still a further embodiment, the recombinant polypeptide is driven by a Plac family promoter.

[0041] In another embodiment, the process involves the use of Pseudomonad host cells that have been genetically modified to provide at least one copy of a LacI encoding gene inserted into the Pseudomonad host cell's genome, wherein the lacI encoding gene is other than as part of the PlacI-lacI-lacZYA operon. In one embodiment, the gene encoding the Lac repressor protein is identical to that of native *E.coli* lacI gene. In another embodiment, the gene encoding the Lac repressor protein is the $lacI^Q$ gene. In still another embodiment, the gene encoding the Lac repressor protein is the $lacI^{Q1}$ gene. In a particular embodiment, the Pseudomonad host cell is *Pseudomonas fluorescens*. In another embodiment, the Pseudomonad is further genetically modified to produce an auxotrophic cell. In another embodiment, the process produces recombinant polypeptide levels of at least about 3 g/L, 4 g/L, 5 g/L 6 g/L, 7 g/L, 8 g/L, 9 g/L or at least about 10 g/L. In another embodiment, the recombinant polypeptide is expressed in levels of between 3 g/L and 100 g/L.

BRIEF DESCRIPTION OF THE FIGURES

[0042] **FIG. 1** represents a comparison of the performance of *P. fluorescens* dual-plasmid expression systems using a pyrF marker (Δ and □) against the performance of *P. fluorescens* dual-plasmid expression systems using only antibiotic resistance markers (♦). All data shown are averages of 9-multiple, representative 20-L fermentations, with IPTG being added to induce target enzyme expression during mid-exponential phase. The upper set of three curves presents relative cell density data, which is read with reference to the left vertical axis. The lower set of three curves presents relative enzyme activity data for the target enzyme produced in the corresponding fermentations, and is read with reference to the right vertical axis. ♦—*P. fluorescens* containing pMYC plasmid having a tac promoter-controlled target enzyme expression cassette and a tetracycline resistance marker gene and containing a pCN plasmid having a lacI repressor expression cassette and a kanamycin resistance marker gene. Variance bars shown are for these data points (n=4), and represent the normal variance typically observed for this expression system among different fermentation runs. Δ—*P. fluorescens* strain with inactivated genomic pyrF containing pMYC plasmid having a tac promoter-controlled target enzyme expression cassette and a pyrF auxotrophic marker gene and containing pCN plasmid having a lacI repressor expression cassette and a kanamycin resistance marker gene. □—*P. fluorescens* strain with inactivated genomic pyrF and proC containing pMYC plasmid having a tac promoter-controlled target enzyme expression cassette and a pyrF auxotrophic marker gene and containing pCN plasmid having a lacI repressor expression cassette and a proC auxotrophic marker gene.

[0043] **FIG. 2** represents a map of the plasmid pDOW1249-2.

[0044] **FIG. 3** represents a map of the plasmid pDOW1269-2.

[0045] **FIG. 4** represents a schematic of lac operator constructs. LacZ represents the positions of the native *E.coli* lacO sequences. tac DC239, DC240 represents the position of the native *E.coli* lac operator on a construct comprising a tac promoter and a nitrilase encoding nucleic acid. Opt lacO DC281 represents the position of the lacOid operator sequence on a construct comprising a tac promoter and a nitrilase encoding nucleic acid. Dual lacO DC262 represents the position of a lacOid operator sequence 5', and wild type lac operator sequence 3' of a tac promoter on a construct further comprising a nitrilase encoding nucleic acid.

[0046] **FIG. 5** represents a Western Blot analysis (UnBlot) of LacI protein accumulation in the lacI integrant strains grown in a shake flask gene expression medium. Broth samples were normalized to OD_{600}, combined with LDS NuPAGE sample buffer (Invitrogen), 50mM DTT and heated at 95° C. for 40 min, then centrifuged briefly. Aliquots of 20 uL were loaded on a 10%, 1 mm NuPAGE Bis-Tris gel run in MOPS with antioxidant in the inner chamber. Detection of the LacI protein was accomplished with an in-gel hybridization method ("UnBlot", Pierce), using a polyclonal rabbit antibody to LacI (Stratagene cat.

no. 217449-51) at 1:1000 and the secondary antibody, Stabilized Goat Anti-rabbit Horseradish Peroxidase Conjugated Antibody (Pierce) at 1:500. The horseradish peroxidase was visualized with UnBlot Stable Peroxide and UnBlot Luminol Enhancer as according to the UnBlot kit.

[0047] **FIG. 6** represents the composite of nitrilase accumulation profiles of DC 140, DC239 and DC240. Data were compiled from DC140 (n=5), DC239 (n=5) and DC240 (n=4) runs. Dc140 is represented by ■. DC239 is represented by □. DC240 is represented by □. Fermentation runs were performed over a 48 hour period.

DETAILED DESCRIPTION OF THE INVENTION

[0048] In one embodiment, the Pseudomonad organisms have been genetically modified to induce an auxotrophy. In a particular embodiment, the Pseudomonad organism is *Pseudomonas fluorescens*. In one embodiment, the auxotrophy is a result of genetic modifications to at least one nitrogenous base compound biosynthesis gene, or at least one amino acid biosynthesis gene. In a further embodiment, the genetic modification is to a gene encoding an enzyme active in the uracil biosynthetic pathway, the thymidine biosynthetic pathway, or the proline biosynthetic pathway. In still a further embodiment, the genetic modification is to the pyrF gene encoding orotidine-5'-phosphate decarboxylase, the thyA gene encoding thymidilate synthase, or the proC gene encoding Δ^1-pyrroline-5-carboxylate reductase.

[0049] In another embodiment, the present invention provides Pseudomonad organisms that have been genetically modified to provide at least one copy of a LacI-encoding gene inserted into the genome, other than as part of the PlacI-lacI-lacZYA operon. In a particular embodiment, the Pseudomonad host cell is *Pseudomonas fluorescens*. In one embodiment, the Pseudomonad contains a native *E.coli* lacI gene encoding the LacI repressor protein.. In another embodiment, the Pseudomonad cell contains the $lacI^Q$ gene. In still another embodiment, the Pseudomonad cell contains the $lacI^{Q1}$ gene.

[0050] In another embodiment, a Pseudomonad organism is provided comprising a nucleic acid construct containing a nucleic acid comprising at least one lacO sequence involved in the repression of transgene expression. In a particular embodiment, the Pseudomonad host cell is *Pseudomonad fluorescens*. In one embodiment, the nucleic acid construct comprises more than one lacO sequence. In another embodiment, the nucleic acid construct comprises at least one, and preferably more than one, lacOid sequence. In one embodiment, the nucleic acid construct comprises a lacO sequence, or derivative thereof, located 3' of a Plac family promoter, and a lacO sequence, or derivative thereof, located 5' of a Plac family promoter. In a particular embodiment, the lacO derivative is a lacOid sequence.

[0051] In a further embodiment, the present invention provides Pseudomonad organisms that have been genetically modified to induce an auxotrophy and further modified to contain a chromosomal insertion of a native *E.coli* lacI gene, lacI Q gene, or lacIQ1 gene other than as part of a whole or truncated Plac-lacI-lacZYA operon. In another embodiment, the Pseudomonad organism is further modified to contain a nucleic acid construct comprising at least one lacO sequence involved in the repression of transgene expression. In a particular embodiment, the Pseudomonad organism is a *Pseudomonas fluorescens*.

[0052] The host cell provided by the present invention for use in an expression system producing recombinant polypeptides can be selected from the "Pseudomonads and closely related bacteria" or from a Subgroup thereof, as defined below. In one embodiment, the host cell is selected from the genus *Pseudomonas*. In a particular embodiment, the particular species of *Pseudomonas* is *P. fluorescens*. In a particular embodiment, the host cell is *Pseudomonas fluorescens* biotype A or biovar I.

[0053] Definitions

[0054] The term "isolated" refers to nucleic acid, protein, or peptide that is substantially or essentially free from other material components, for example, which can be cellular components.

[0055] The term "fragment" means a portion or partial sequence of a nucleotide, protein, or peptide sequence.

[0056] As used herein, the term "percent total cell protein" means the amount of protein or peptide in the host cell as a percentage of aggregate cellular protein.

[0057] The term "operably attached," as used herein, refers to any configuration in which the transcriptional and any translational regulatory elements are covalently attached to the encoding sequence in such disposition(s), relative to the coding sequence, that in and by action of the host cell, the regulatory elements can direct the expression of the coding sequence.

[0058] The term "auxotrophic," as used herein, refers to a cell which has been modified to eliminate or reduce its ability to produce a specific substance required for growth and metabolism.

[0059] As used herein, the term "percent total cell protein" means a measure of the fraction of total cell protein that represents the relative amount of a given protein expressed by the cell.

[0060] The term "prototrophy," as used herein, refers to a cell that is capable of producing a specific substance required for growth and metabolism.

[0061] As used herein, the term "homologous" or means either i) a protein or peptide that has an amino acid sequence that is substantially similar (i.e., at least 70, 75, 80, 85, 90, 95, or 98%) to the sequence of a given original protein or peptide and that retains a desired function of the original protein or peptide or ii) a nucleic acid that has a sequence that is substantially similar (i.e., at least 70, 75, 80, 5, 90, 95, or 98%) to the sequence of a given nucleic acid and that retains a desired function of the original nucleic acid sequence. In all of the embodiments of this invention and disclosure, any disclosed protein, peptide or nucleic acid can be substituted with a homologous or substantially homologous protein, peptide or nucleic acid that retains a desired function. In all of the embodiments of this invention and disclosure, when any nucleic acid is disclosed, it should be assumed that the invention also includes all nucleic acids that hybridize to the disclosed nucleic acid.

[0062] In one non-limiting embodiment, the non-identical amino acid sequence of the homologous polypeptide can be

amino acids that are members of any one of the 15 conservative or semi-conservative groups shown in Table 1.

TABLE 1

SIMILAR AMINO ACID SUBSTITUTION GROUPS

Conservative Groups (8)	Semi-Conservative Groups (7)
Arg, Lys	Arg, Lys, His
Asp, Glu	Asn, Asp, Glu, Gln
Asn, Gln	
Ile, Leu, Val	Ile, Leu, Val, Met, Phe
Ala, Gly	Ala, Gly, Pro, Ser, Thr
Ser, Thr	Ser, Thr, Tyr
Phe, Tyr	Phe, Trp, Tyr
Cys (non-cystine), Ser	Cys (non-Cystine), Ser, Thr

[0063] Amino acid sequences provided herein are represented by the following abbreviations:

A	Ala	alanine
P	Pro	proline
B		aspartate or asparagine
Q	Gln	glutamine
C	Cys	cysteine
R	Arg	arginine
D	Asp	aspartate
S	Ser	serine
E	Glu	glutamate
T	Thr	threonine
F	Phe	phenylalanine
G	Gly	glycine
V	Val	valine
H	His	histidine
W	Trp	tryptophan
I	Ile	isoleucine
Y	Tyr	tyrosine
Z		glutamate or glutamine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine

[0064] I. Selection of Pseudomonads and Related Bacteria as Host Cells

[0065] The present invention provides the use of Pseudomonads and related bacteria as host cells in the improved production of proteins.

[0066] Auxotrophic Selection Efficiency

[0067] It has been discovered that Pseudomonads have the ability to utilize auxotrophic selection markers for the maintenance of protein expressing plasmids without the drawbacks typically associated with other systems, such as plasmid instability and cross-feeding.

[0068] Auxotrophic markers, in other host cell systems, are not always sufficient to maintain plasmids in every cell, especially during fermentations where loss of the plasmid may give plasmid-less cells a selective advantage, resulting in the accumulation of a large fraction of nonproductive cells, reducing product formation. Such revertant strains are especially troublesome if they have the ability to cross-feed the auxotrophic metabolite from prototrophic enabled bacteria. For example, use of the trp operon on a plasmid in an *E.coli* tryptophan auxotroph was not sufficient to prevent a large proportion of plasmid-less cells from accumulating, until combined with the valS gene (encoding valyl t-RNA synthetase) in a $valS^{ts}$ host (Skogman, S. G.; Nilsson, J., Temperature-dependent retention of a tryptophan-operon-bearing plasmid in *Escherichia coli*. *Gene* 1984, 31, (1-3), 117-22.) Presumably, the cells containing the trp operon on a plasmid secreted enough tryptophan or related molecules to allow growth of plasmid-less cells. Likewise, using the LEU2 gene on a xylitol-reductase production plasmid in leu2 mutant yeast resulted in plasmid loss; up to 80% of a fed-batch culture was made up of cells without a production plasmid, because leucine was secreted by plasmid-containing cells into the broth (Meinander, N. Q.; Hahn-Haegerdal, B., Fed-batch xylitol production with two recombinant *Saccharomyces cerevisiae* strains expressing XYL1 at different levels, using glucose as a cosubstrate: a comparison of production parameters and strain stability. *Biotechnology and Bioengineering* 1997, 54, (4), 391-399).

[0069] It has been discovered that *Pseudomonas fluorescens* (Pf) does not exhibit the inherent problems associated with cross-feeding observed in other host cell systems, for example, *E.coli* and yeast. While not wanting to be bound by any particular theory, it is thought that auxotrophic *Pseudomonas fluorescens* is a particularly suitable organism for use as a host cell because of the observed inability of a Pf auxotrophic cell to out compete a auxotrophic cell containing a prototrophic-enabling plasmid on a supplemented medium that contains the auxotrophic metabolite, indicating an innate difficulty of an Pf auxotroph to import the required metabolite. Because of this, Pf auxotrophic cells that lose the selection marker plasmid do not gain a selective advantage over Pf auxotrophic cells containing the selection marker, even in the presence of a supplemental metabolite, greatly reducing any potential effects of cross-feeding. Because of the reduced effects of cross-feeding, production yields of the recombinant polypeptide in a fermentation run are not reduced due to the presence of non-recombinant polypeptide producing cells.

[0070] LacI Insert

[0071] It has been discovered that Pseudomonads are able to use a single-copy lacI transgene, other than as part of a whole or truncated Plac-lacI-lacZYA operon, chromosomal insert to effectively repress protein expression until induction.

[0072] Transcription initiation from regulated promoters by RNA polymerase is activated or deactivated by the binding or releasing of a regulatory protein. Thus, regulated promoters include those that participate in negative control (i.e. repressible promoters), wherein the gene encoding the target polypeptide of interest is expressed only when the promoter is free of the regulator protein (i.e. a "repressor" protein), and those that participate in positive control, wherein the gene is expressed only when the promoter is bound by the regulator protein (i.e. an "activator" protein).

[0073] One of the most common classes of repressible promoters used in bacterial expression systems is the family of Plac-based promoters. The family of Plac-based promoters originates with the native *E.coli* lactose operon, referred to as the "lac" operon, also symbolized as "lacZYA," the expression of which is regulated by the expression product of the lacI gene. The native *E.coli* structure of the operon is "PlacI-lacI-PlacZ-lacZYA," wherein the native *E.coli* Plac

promoter is represented by "PlacZ" (also called "PlacZYA"). "PlacI" represents the native promoter for the lacI gene, and "lacI" represents the gene encoding the lac repressor, i.e. the LacI protein. "lacZYA" represents the operon encoding the lactose utilization pathway.

[0074] The LacI-regulated promoters include, among others, the native *E.coli* lactose operon promoter ("Plac"). In addition, improved mutants have also been discovered, as have intra promoter hybrids of Plac, such as the "Ptac" promoter, "Ptrc" promoter, and the "PtacII" promoters. The Ptac promoter in *E.coli,* for example, is 3-fold stronger than the Plac promoter when fully derepressed. Therefore, it is frequently used for promoting high level regulated gene expression in *E.coli*. However, while the Plac promoter is 1,000-fold repressed by LacI, while the Ptac promoter is only 50-fold repressed under similar conditions (Lanzer, M. & H. Bujard. 1988. Proc. Natl. Acad. Sci. USA. 85:8973). Repression of the *E. coli* Ptac promoter or other lac related promoters, depends upon the concentration of the repressor, LacI. (De Boer, et al., 1983. Proc. Natl. Acad. Sci. USA. 78:21-25). As set forth above, release from repression can occur through the addition of an inducer which reduces the affinity of the repressor for its specific DNA binding site, in this case, the lac operator (lacO). Alternatively, a reduction in the concentration of the repressor relative to the molar concentration of specific DNA binding sites on the plasmid can also derepress the promoter. If the lacI gene is located on a high copy number cloning plasmid, then a large amount of inducer is required to initiate expression because of the large amount of repressor produced in such a system.

[0075] In commercial production systems, the lac repressor is typically encoded by a gene whose expression is constitutive, i.e. non-regulated, thus providing an intracellular environment in which the desired transgene, encoding the desired target protein, is repressed until a desired host cell biomass or cell density is achieved. At that time, a quantity of a small molecule known as an inducer whose presence is effective to dissociate the repressor from the transgene, is added to the cell culture and taken up by the host cell, thereby permitting transcription of the transgene. In the case of lac repressor proteins, the inducer can be lactose or a non-metabolized, gratuitous inducer such as isopropyl-beta-D-thio-galactoside ("IPTG"). The selected point in time at which the inducer is to be added is referred to as the "induction phase."

[0076] A variety of lac repressor genes have been identified as useful for the repression of Plac family promoters present on recombinant polypeptide expression vectors. These include the native *E.coli* lacI gene and/or by variants thereof, including the $lacI^{Q}$ and $lacI^{Q1}$ genes that encode the same LacI protein, but at a higher expression level. For example, the $lacI^{Q}$ mutation is a single CG to TA change at -35 of the promoter region of lacI (Calos, M. 1978. Nature 274:762) which causes a 10-fold increase in LacI expression in *E.coli* (Mueller-Hill, B., et al. 1968. Proc. Natl. Acad. Sci. USA. 59:1259). Wild-type *E.coli* cells have a concentration of LacI of 10^{-8} M or about 10 molecules per cell, with 99% of the protein present as a tetramer (Fickert, R. & B. Mueller-Hill 1992. J. Mol. Biol. 226:59). Cells containing the $lacI^{Q}$ mutation contain about 100 molecules per cell or 10^{-7} M LacI. As a result, a number of bacterial expression systems have been developed in which Plac family promoter controlled transgenes, resident in plasmids, are maintained in host cells expressing LacI proteins at different levels, thereby repressing the desired transgene until a chosen "induction phase" of cell growth.

[0077] In many cases, however, repression of expression of the target protein during cell growth can be imperfect, resulting in a significant amount of expression prior to the particular induction phase. This "leaky" repression results in host cell stress, inefficient utilization of carbon source due to metabolic energy being diverted from normal cell growth to transgene, and a delay in reaching optimal cell density induction points, resulting in a more lengthy and costly fermentation run, and often, a reduced yield of the target protein.

[0078] One common strategy for improving repression of Plac-family promoter-driven transgenes has been to place a lacI or a $lacI^{Q}$ gene on the plasmid bearing the Plac-family promoter-driven target gene (e.g. see MJR Stark in Gene 51:255-67 (1987) and E Amann et al. in Gene:301-15 (1988)). However, this often results in overproduction of the Lac repressor protein, which then requires use of an even higher inducer concentration to restore induction levels of the transgene to overcome the decrease in recombinant protein production. Moreover, the use of a second plasmid containing the lacI gene, separate from the plasmid containing the Plac-family promoter-driven target gene, requires the use of two different selection marker genes in order to maintain both plasmids in the expression host cell: one selection marker gene for each of the two different plasmids. The presence of the second selection marker gene, i.e. the selection marker gene for the second plasmid, in turn requires the use of either: 1) a separate antibiotic in the case of an antibiotic-resistance selection marker gene, which is costly and disadvantageous from a health/safety regulatory perspective; or 2) a separate metabolic deficiency in the host cell genome, in the case of an auxotrophic selection marker gene, which requires the additional work of mutating the host cell.

[0079] It has surprisingly been discovered that a lacI insertion, other than as part of a whole or truncated PlacI-lacI-lacZYA operon, is as effective in repressing Plac-Ptac family promoter-controlled transgenes as a multi-copy plasmid encoding a LacI repressor protein in *Pseudomonas fluorescens*. This surprising discovery eliminates the need to maintain a separate plasmid encoding a LacI repressor protein in the cell, or eliminates the need to define an additional auxotrophic selection marker, and further reduces the potential production inefficiencies caused by such maintenance of a lacI containing plasmid.

[0080] In a previous attempt to regulate transgene expression in *Pseudomonas,* an *E.coli* PlacI-lacI-lacZYA operon that has been deleted of the lacZ promoter region, but that retains the constitutive PlacI promoter, was chromosomally inserted (See U.S. Pat. No. 5,169,760). The deletion allows for constitutive expression of the gene products of the lac operon. However, the inserted operon contains the *E.coli* lacy gene, which encodes for the lactose transporter protein lactose permease. Lactose permease is capable of transporting lactose, or similar derivatives, into the host cell from the medium. The presence of lactose permease may lead to increased importation of lactose-like contaminants from the medium, ultimately resulting in derepression of the Plac family promoter prior to induction. Furthermore, expression

of the lac operon lacZ, lacY, and lacA gene products may result in the inefficient dedication of carbon utilization resources to these products, resulting in increased metabolic stress on the cells, and delaying the establishment of a high cell density for induction. In addition, the larger lacI-lacZYA fusion operon may produce increased message instability compared to a lacI insert alone in a host cell.

[0081] It has been surprisingly discovered that the use of a LacI-encoding gene other than as part of a whole or truncated PlacI-lacI-lacZYA operon in Pseudomonads surprisingly resulted in substantially improved repression of pre-induction recombinant protein expression, higher cell densities in commercial-scale fermentation, and higher yields of the desired product in comparison with previously taught lacI-lacZYA Pseudomonad chromosomal insertion (U.S. Pat. No. 5,169,760).

[0082] Additional attempts to utilize derivative lacI genes, such as $lacI^{Q}$ and $lacI^{Q1,}$ which are expressed at greater levels than lacI due to promoter modifications, have also been described. C G Glascock & M J Weickert describe *E.coli* strains in which a separate LacI protein-encoding gene was present in the chromosome of the host cell in an attempt to assess the level of control of a plasmid-borne Ptac-driven target gene. See C G Glascock & M J Weickert, "Using chromosomal $lacI^{Q1}$ to control expression of genes on high-copy number plasmids in *Escherichia coli*," Gene 223(1-2):221-31(1998); See also WO 97/04110. Among the LacI protein-encoding genes tested were lacI, $lacI^{Q}$, and $lacI^{Q1}$. The results obtained for the lacI gene and the $lacI^{Q}$ gene demonstrated inferior levels of repression of the Ptac-driven target gene when present on a high-copy number plasmid, resulting in substantial levels of pre-induction target gene expression. Only the high expressing $lacI^{Q1}$ gene provided sufficient repression in that system.

[0083] Such a strategy, however, has the potential to increase costs by increasing the amount of inducer required to sufficiently derepress the promoter at induction, and decreasing yields due to the inability of the inducer to sufficiently bind all of the constitutively expressed repressor protein.

[0084] Comparatively, it has surprisingly been discovered that a single-copy lacI chromosomal insert was sufficient to repress Plac-Ptac family promoter driven transgene expression. Such a discovery allows potential cost saving measures on the amount of inducer used, and provides additional flexibility in the development of *Pseudomonas fluorescens* as a host cell in the improved production of proteins.

[0085] *Pseudomonas* Organisms

[0086] Pseudomonads and closely related bacteria, as used herein, is co-extensive with the group defined herein as "Gram(−) Proteobacteria Subgroup 1."Gram(−) Proteobacteria Subgroup 1" is more specifically defined as the group of Proteobacteria belonging to the families and/or genera described as falling within that taxonomic "Part" named "Gram-Negative Aerobic Rods and Cocci" by R. E. Buchanan and N. E. Gibbons (eds.), Bergey's Manual of Determinative Bacteriology, pp. 217-289 (8th ed., 1974) (The Williams & Wilkins Co., Baltimore, Md., USA) (hereinafter "Bergey (1974)"). Table 4 presents the families and genera of organisms listed in this taxonomic "Part."

TABLE 1

FAMILIES AND GENERA LISTED IN THE PART, "GRAM-NEGATIVE AEROBIC RODS AND COCCI" (IN BERGEY (1974))

Family I. Pseudomonadaceae	*Gluconobacter*
	Pseudomonas
	Xanthomonas
	Zoogloea
Family II. Azotobacteraceae	*Azomonas*
	Azotobacter
	Beijerinckia
	Derxia
Family III. Rhizobiaceae	*Agrobacterium*
	Rhizobium
Family IV. Methylomonadaceae	*Methylococcus*
	Methylomonas
Family V. Halobacteriaceae	*Halobacterium*
	Halococcus
Other Genera	*Acetobacter*
	Alcaligenes
	Bordetella
	Brucella
	Francisella
	Thermus

[0087] "Gram(−) Proteobacteria Subgroup 1"contains all Proteobacteria classified there under, as well as all Proteobacteria that would be classified according to the criteria used in forming that taxonomic "Part." As a result, "Gram(−) Proteobacteria Subgroup 1" excludes, e.g.: all Gram-positive bacteria; those Gram-negative bacteria, such as the Enterobacteriaceae, which fall under others of the 19 "Parts" of this Bergey (1974) taxonomy; the entire "Family V. Halobacteriaceae" of this Bergey (1974) "Part," which family has since been recognized as being a non-bacterial family of Archaea; and the genus, *Thermus*, listed within this Bergey (1974) "Part," which genus which has since been recognized as being a non-Proteobacterial genus of bacteria. "Gram(−) Proteobacteria Subgroup 1" further includes those Proteobacteria belonging to (and previously called species of) the genera and families defined in this Bergey (1974) "Part," and which have since been given other Proteobacterial taxonomic names. In some cases, these re-namings resulted in the creation of entirely new Proteobacterial genera. For example, the genera *Acidovorax, Brevundimonas, Burkholderia, Hydrogenophaga, Oceanimonas, Ralstonia,* and *Stenotrophomonas,* were created by regrouping organisms belonging to (and previously called species of) the genus *Pseudomonas* as defined in Bergey (1974). Likewise, e.g., the genus *Sphingomonas* (and the genus *Blastomonas,* derived therefrom) was created by regrouping organisms belonging to (and previously called species of) the genus *Xanthomonas* as defined in Bergey (1974). Similarly, e.g., the genus *Acidomonas* was created by regrouping organisms belonging to (and previously called species of) the genus *Acetobacter* as defined in Bergey (1974). Such subsequently reassigned species are also included within "Gram(−) Proteobacteria Subgroup 1" as defined herein.

[0088] In other cases, Proteobacterial species falling within the genera and families defined in this Bergey (1974) "Part" were simply reclassified under other, existing genera of Proteobacteria. For example, in the case of the genus *Pseudomonas, Pseudomonas enalia* (ATCC 14393), *Pseudomonas nigrifaciens* (ATCC 19375), and *Pseudomonas putrefaciens* (ATCC 8071) have since been reclassified respectively as *Alteromonas haloplanktis, Alteromonas*

nigrifaciens, and *Alteromonas putrefaciens*. Similarly, e.g., *Pseudomonas acidovorans* (ATCC 15668) and *Pseudomonas testosteroni* (ATCC 11996) have since been reclassified as *Comamonas acidovorans* and *Comamonas testosteroni,* respectively; and *Pseudomonas nigrifaciens* (ATCC 19375) and *Pseudomonas piscicida* (ATCC 15057) have since been reclassified respectively as *Pseudoalteromonas nigrifaciens* and *Pseudoalteromonas piscicida.* Such subsequently reassigned Proteobacterial species are also included within "Gram(−) Proteobacteria Subgroup 1" as defined herein. "Gram(−) Proteobacteria Subgroup 1" also includes Proteobacterial species that have since been discovered, or that have since been reclassified as belonging, within the Proteobacterial families and/or genera of this Bergey (1974) "Part." In regard to Proteobacterial families, "Gram(−) Proteobacteria Subgroup 1" also includes Proteobacteria classified as belonging to any of the families: Pseudomonadaceae, Azotobacteraceae (now often called by the synonym, the "Azotobacter group" of Pseudomonadaceae), Rhizobiaceae, and Methylomonadaceae (now often called by the synonym, "Methylococcaceae"). Consequently, in addition to those genera otherwise described herein, further Proteobacterial genera falling within "Gram(−) Proteobacteria Subgroup 1" include: 1) Azotobacter group bacteria of the genus *Azorhizophilus;* 2) Pseudomonadaceae family bacteria of the genera *Cellvibrio, Oligella,* and *Teredinibacter;* 3) Rhizobiaceae family bacteria of the genera *Chelatobacter, Ensifer, Liberibacter* (also called "*Candidatus Liberibacter*"), and *Sinorhizobium;* and 4) Methylococcaceae family bacteria of the genera *Methylobacter, Methylocaldum, Methylomicrobium, Methylosarcina,* and *Methylosphaera.*

[0089] In one embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 1,"as defined above.

[0090] In another embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 2.""Gram(−) Proteobacteria Subgroup 2" is defined as the group of Proteobacteria of the following genera (with the total numbers of catalog-listed, publicly-available, deposited strains thereof indicated in parenthesis, all deposited at ATCC, except as otherwise indicated): *Acidomonas* (2); *Acetobacter* (93); *Gluconobacter* (37); *Brevundimonas* (23); *Beijerinckia* (13); *Derxia* (2); *Brucella* (4); *Agrobacterium* (79); *Chelatobacter* (2); *Ensifer* (3); *Rhizobium* (144); *Sinorhizobium* (24); *Blastomonas* (1); *Sphingomonas* (27); *Alcaligenes* (88); *Bordetella* (43); *Burkholderia* (73); *Ralstonia* (33); *Acidovorax* (20); *Hydrogenophaga* (9); *Zoogloea* (9); *Methylobacter* (2); *Methylocaldum* (1 at NCIMB); *Methylococcus* (2); *Methylomicrobium* (2); *Methylomonas* (9); *Methylosarcina* (1); *Methylosphaera; Azomonas* (9); *Azorhizophilus* (5); *Azotobacter* (64); *Cellvibrio* (3); *Oligella* (5); *Pseudomonas* (1139); *Francisella* (4); *Xanthomonas* (229); *Stenotrophomonas* (50); and *Oceanimonas* (4).

[0091] Exemplary host cell species of "Gram(−) Proteobacteria Subgroup 2" include, but are not limited to the following bacteria (with the ATCC or other deposit numbers of exemplary strain(s) thereof shown in parenthesis): *Acidomonas methanolica* (ATCC 43581); *Acetobacter aceti* (ATCC 15973); *Gluconobacter oxydans* (ATCC 19357); *Brevundimonas diminuta* (ATCC 11568); *Bejerinckia indica* (ATCC 9039 and ATCC 19361); *Derxia gummosa* (ATCC 15994); *Brucella melitensis* (ATCC 23456), *Brucella abortus* (ATCC 23448); *Agrobacterium tumefaciens* (ATCC 23308), *Agrobacterium radiobacter* (ATCC 19358), *Agrobacterium rhizogenes* (ATCC 11325); *Chelatobacter heintzii* (ATCC 29600); *Ensifer adhaerens* (ATCC 33212); *Rhizobium leguminosarum* (ATCC 10004); *Sinorhizobium fredii* (ATCC 35423); *Blastomonas natatoria* (ATCC 35951); *Sphingomonas paucimobilis* (ATCC 29837); *Alcaligenes faecalis* (ATCC 8750); *Bordetella pertussis* (ATCC 9797); *Burkholderia cepacia* (ATCC 25416); *Ralstonia pickettii* (ATCC 27511); *Acidovoraxfacilis* (ATCC 11228); *Hydrogenophaga flava* (ATCC 33667); *Zoogloea ramigera* (ATCC 19544); *Methylobacter luteus* (ATCC 49878); *Methylocaldum gracile* (NCIMB 11912); *Methylococcus capsulatus* (ATCC 19069); *Methylomicrobium agile* (ATCC 35068); *Methylomonas methanica* (ATCC 35067); *Methylosarcina fibrata* (ATCC 700909); *Methylosphaera hansonii* (ACAM 549); *Azomonas agilis* (ATCC 7494); *Azorhizophilus paspali* (ATCC 23833); *Azotobacter chroococcum* (ATCC 9043); *Cellvibrio mixtus* (UQM 2601); *Oligella urethralis* (ATCC 17960); *Pseudomonas aeruginosa* (ATCC 10145), *Pseudomonas fluorescens* (ATCC 35858); *Francisella tularensis* (ATCC 6223); *Stenotrophomonas maltophilia* (ATCC 13637); *Xanthomonas campestris* (ATCC 33913); and *Oceanimonas doudoroffii* (ATCC 27123).

[0092] In another embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 3.""Gram(−) Proteobacteria Subgroup 3" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Agrobacterium; Rhizobium; Sinorhizobium; Blastomonas; Sphingomonas ; Alcaligenes; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Methylobacter; Methylocaldum; Methylococcus; Methylomicrobium; Methylomonas; Methylosarcina; Methylosphaera; Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas; Teredinibacter; Francisella; Stenotrophomonas; Xanthomonas;* and *Oceanimonas.*

[0093] In another embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 4.""Gram(−) Proteobacteria Subgroup 4" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Blastomonas; Sphingomonas; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Methylobacter; Methylocaldum; Methylococcus; Methylomicrobium; Methylomonas; Methylosarcina; Methylosphaera; Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas; Teredinibacter; Francisella; Stenotrophomonas; Xanthomonas;* and *Oceanimonas.*

[0094] In an embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 5.""Gram(−) Proteobacteria Subgroup 5" is defined as the group of Proteobacteria of the following genera: *Methylobacter; Methylocaldum; Methylococcus; Methylomicrobium; Methylomonas; Methylosarcina; Methylosphaera; Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas; Teredinibacter; Francisella; Stenotrophomonas; Xanthomonas;* and *Oceanimonas.*

[0095] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 6.""Gram(−) Proteobacteria Subgroup 6" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Blastomonas; Sphingomonas; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas; Teredinibacter; Stenotrophomonas; Xanthomonas;* and *Oceanimonas.*

[0096] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 7.""Gram(−) Proteobacteria Subgroup 7" is defined as the group of Proteobacteria of the following genera: *Azomonas; Azorhizophilus; Azotobacter; Cellvibrio; Oligella; Pseudomonas; Teredinibacter; Stenotrophomonas; Xanthomonas;* and *Oceanimonas.*

[0097] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 8.""Gram(−) Proteobacteria Subgroup 8" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Blastomonas; Sphingomonas; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Pseudomonas; Stenotrophomonas; Xanthomonas;* and *Oceanimonas.*

[0098] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 9.""Gram(−) Proteobacteria Subgroup 9" is defined as the group of Proteobacteria of the following genera: *Brevundimonas; Burkholderia; Ralstonia; Acidovorax; Hydrogenophaga; Pseudomonas; Stenotrophomonas;* and *Oceanimonas.*

[0099] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 10.""Gram(−) Proteobacteria Subgroup 10" is defined as the group of Proteobacteria of the following genera: *Burkholderia; Ralstonia; Pseudomonas; Stenotrophomonas;* and *Xanthomonas.*

[0100] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 11.""Gram(−) Proteobacteria Subgroup 11" is defined as the group of Proteobacteria of the genera: *Pseudomonas; Stenotrophomonas;* and *Xanthomonas.*

[0101] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 12.""Gram(−) Proteobacteria Subgroup 12" is defined as the group of Proteobacteria of the following genera: *Burkholderia; Ralstonia; Pseudomonas.*

[0102] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 13.""Gram(−) Proteobacteria Subgroup 13" is defined as the group of Proteobacteria of the following genera: *Burkholderia; Ralstonia; Pseudomonas;* and *Xanthomonas.*

[0103] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 14.""Gram(−) Proteobacteria Subgroup 14" is defined as the group of Proteobacteria of the following genera: *Pseudomonas* and *Xanthomonas.*

[0104] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 15.""Gram(−) Proteobacteria Subgroup 15" is defined as the group of Proteobacteria of the genus *Pseudomonas.*

[0105] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 16. ""Gram(−) Proteobacteria Subgroup 16" is defined as the group of Proteobacteria of the following Pseudomonas species (with the ATCC or other deposit numbers of exemplary strain(s) shown in parenthesis): *Pseudomonas abietaniphila* (ATCC 700689); *Pseudomonas aeruginosa* (ATCC 10145); *Pseudomonas alcaligenes* (ATCC 14909); *Pseudomonas anguilliseptica* (ATCC 33660); *Pseudomonas citronellolis* (ATCC 13674); *Pseudomonasflavescens* (ATCC 51555); *Pseudomonas mendocina* (ATCC 25411); *Pseudomonas nitroreducens* (ATCC 33634); *Pseudomonas oleovorans* (ATCC 8062); *Pseudomonas pseudoalcaligenes* (ATCC 17440); *Pseudomonas resinovorans* (ATCC 14235); *Pseudomonas straminea* (ATCC 33636); *Pseudomonas agarici* (ATCC 25941); *Pseudomonas alcaliphila; Pseudomonas alginovora; Pseudomonas andersonii; Pseudomonas asplenii* (ATCC 23835); *Pseudomonas azelaica* (ATCC 27162); *Pseudomonas beijerinckii* (ATCC 19372); *Pseudomonas borealis; Pseudomonas boreopolis* (ATCC 33662); *Pseudomonas brassicacearum; Pseudomonas butanovora* (ATCC 43655); *Pseudomonas cellulosa* (ATCC 55703); *Pseudomonas aurantiaca* (ATCC 33663); *Pseudomonas chlororaphis* (ATCC 9446, ATCC 13985, ATCC 17418, ATCC 17461); *Pseudomonas fragi* (ATCC 4973); *Pseudomonas lundensis* (ATCC 49968); *Pseudomonas taetrolens* (ATCC 4683); *Pseudomonas cissicola* (ATCC 33616); *Pseudomonas coronafaciens; Pseudomonas diterpeniphila; Pseudomonas elongata* (ATCC 10144); *Pseudomonas flectens* (ATCC 12775); *Pseudomonas azotoformans; Pseudomonas brenneri; Pseudomonas cedrella; Pseudomonas corrugata* (ATCC 29736); *Pseudomonas extremorientalis; Pseudomonas fluorescens* (ATCC 35858); *Pseudomonas gessardii; Pseudomonas libanensis; Pseudomonas mandelii* (ATCC 700871); *Pseudomonas marginalis* (ATCC 10844); *Pseudomonas migulae; Pseudomonas mucidolens* (ATCC 4685); *Pseudomonas orientalis; Pseudomonas rhodesiae; Pseudomonas synxantha* (ATCC 9890); *Pseudomonas tolaasii* (ATCC 33618); *Pseudomonas veronii* (ATCC 700474); *Pseudomonas frederiksbergensis; Pseudomonas geniculata* (ATCC 19374); *Pseudomonas gingeri; Pseudomonas graminis; Pseudomonas grimontii; Pseudomonas halodenitrificans; Pseudomonas halophila; Pseudomonas hibiscicola* (ATCC 19867); *Pseudomonas huttiensis* (ATCC 14670); *Pseudomonas hydrogenovora; Pseudomonas jessenii* (ATCC 700870); *Pseudomonas kilonensis; Pseudomonas lanceolata* (ATCC 14669); *Pseudomonas lini; Pseudomonas marginata* (ATCC 25417); *Pseudomonas mephitica* (ATCC 33665); *Pseudomonas denitrificans* (ATCC 19244); *Pseudomonas pertucinogena* (ATCC 190); *Pseudomonas pictorum* (ATCC 23328); *Pseudomonas psychrophila; Pseudomonas fulva* (ATCC 31418); *Pseudomonas monteilii* (ATCC 700476); *Pseudomonas mosselii; Pseudomonas oryzihabitans* (ATCC 43272); *Pseudomonas plecoglossicida* (ATCC 700383); *Pseudomonas putida* (ATCC 12633); *Pseudomonas reactans; Pseudomonas spinosa* (ATCC 14606); *Pseudomonas balearica; Pseudomonas luteola* (ATCC 43273); *Pseudomonas stutzeri* (ATCC 17588); *Pseudomonas amygdali* (ATCC 33614); *Pseudomonas avellanae* (ATCC 700331); *Pseudomonas caricapapayae* (ATCC 33615); *Pseudomonas cichorii* (ATCC 10857); *Pseudomonas fcuserectae* (ATCC 35104); *Pseudomonas fuscovaginae; Pseudomonas meliae* (ATCC 33050); *Pseudomonas syringae* (ATCC 19310); *Pseudomonas viridiflava* (ATCC 13223); *Pseudomonas thermocarboxydovorans* (ATCC 35961); *Pseudomonas thermotolerans; Pseudomonas thivervalensis; Pseudomonas vancouverensis* (ATCC 700688); *Pseudomonas wisconsinensis;* and *Pseudomonas xiamenensis.*

[0106] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 17.""Gram(−) Proteobacteria Subgroup 17" is defined as the group of Proteobacteria known in the art as the "fluorescent Pseudomonads" including those belonging, e.g., to the following *Pseudomonas* species: *Pseudomonas azotoformans; Pseudomonas brenneri; Pseudomonas cedrella; Pseudomonas corrugata; Pseudomonas extremorientalis; Pseudomonas fluorescens; Pseudomonas gessardii; Pseudomonas libanensis;*

Pseudomonas mandelii; Pseudomonas marginalis; Pseudomonas migulae; Pseudomonas mucidolens; Pseudomonas orientalis; Pseudomonas rhodesiae; Pseudomonas synxantha; Pseudomonas tolaasii; and *Pseudomonas veronii.*

[0107] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 18.""Gram(−) Proteobacteria Subgroup 18" is defined as the group of all subspecies, varieties, strains, and other sub-special units of the species *Pseudomonas fluorescens,* including those belonging, e.g., to the following (with the ATCC or other deposit numbers of exemplary strain(s) shown in parenthesis): *Pseudomonas fluorescens* biotype A, also called biovar 1 or biovar I (ATCC 13525); *Pseudomonas fluorescens* biotype B, also called biovar 2 or biovar II (ATCC 17816); *Pseudomonas fluorescens* biotype C, also called biovar 3 or biovar III (ATCC 17400); *Pseudomonas fluorescens* biotype F, also called biovar 4 or biovar IV (ATCC 12983); *Pseudomonas fluorescens* biotype G, also called biovar 5 or biovar V (ATCC 17518); *Pseudomonas fluorescens* biovar VI; *Pseudomonas fluorescens* Pf0-1; *Pseudomonas fluorescens* Pf-5 (ATCC BAA-477); *Pseudomonas fluorescens* SBW25; and *Pseudomonas fluorescens* subsp. cellulosa (NCIMB 10462).

[0108] The host cell can be selected from "Gram(−) Proteobacteria Subgroup 19.""Gram(−) Proteobacteria Subgroup 19" is defined as the group of all strains of *Pseudomonas fluorescens* biotype A. A particularly particular strain of this biotype is *P. fluorescens* strain MB101 (see U.S. Pat. No. 5,169,760 to Wilcox), and derivatives thereof.

[0109] In one embodiment, the host cell is any of the Proteobacteria of the order Pseudomonadales. In a particular embodiment, the host cell is any of the Proteobacteria of the family Pseudomonadaceae.

[0110] In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 1. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 2. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 3. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 5. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 7. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 12. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 15. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 17. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 18. " In a particular embodiment, the host cell is selected from "Gram(−) Proteobacteria Subgroup 19. "

[0111] Additional *P. fluorescens* strains that can be used in the present invention include *Pseudomonas fluorescens* Migula and *Pseudomonas fluorescens* Loitokitok, having the following ATCC designations: [NCIB 8286]; NRRL B-1244; NCIB 8865 strain COI; NCIB 8866 strain CO2; 1291 [ATCC 17458; IFO 15837; NCIB 8917; LA; NRRL B-1864; pyrrolidine; PW2 [ICMP 3966; NCPPB 967; NRRL B-899]; 13475; NCTC 10038; NRRL B-1603 [6; IFO 15840]; 52-1C; CCEB 488-A [BU 140]; CCEB 553 [IEM 15/47]; IAM 1008 [AHH-27]; IAM 1055 [AHH-23]; 1 [IFO 15842]; 12 [ATCC 25323; NIH 11; den Dooren de Jong 216]; 18 [IFO 15833; WRRL P-7]; 93 [TR-10]; 108[52-22; IFO 15832]; 143 [IFO 15836; PL]; 149 [2-40-40; IFO 15838]; 182 [IFO 3081; PJ 73]; 184 [IFO 15830]; 185[W2 L-1]; 186 [IFO 15829; PJ 79]; 187 [NCPPB 263]; 188 [NCPPB 316]; 189 [PJ227; 1208]; 191 [IFO 15834; PJ 236; 22/1]; 194 [Klinge R-60; PJ 253]; 196 [PJ 288]; 197 [PJ 290]; 198[PJ 302]; 201 [PJ 368]; 202 [PJ 372]; 203 [PJ 376]; 204 [IFO 15835; PJ 682]; 205[PJ686]; 206 [PJ 692]; 207 [PJ 693]; 208 [PJ 722]; 212 [PJ 832]; 215 [PJ 849]; 216 [PJ885]; 267 [B-9]; 271 [B-1612]; 401 [C71A; IFO 15831; PJ 187]; NRRL B-3178 [4; IFO 15841]; KY8521; 3081; 30-21; [IFO 3081]; N; PYR; PW; D946-B83 [BU 2183; FERM-P 3328]; P-2563 [FERM-P 2894; IFO 13658]; IAM-1126 [43F]; M-1; A506 [A5-06]; A505[A5-05-1]; A526 [A5-26]; B69; 72; NRRL B4290; PMW6 [NCIB 11615]; SC 12936; Al [IFO 15839]; F 1847 [CDC-EB]; F 1848 [CDC 93]; NCIB 10586; P17; F-12; AmMS 257; PRA25; 6133D02; 6519E01; Ni; SC15208; BNL-WVC; NCTC 2583 [NCIB 8194]; H13; 1013 [ATCC 11251; CCEB 295]; IFO 3903; 1062; or Pf-5.

II. Auxotrophic Selection Markers

[0112] The present invention provides Pseudomonads and related cells that have been genetically modified to induce auxotrophy for at least one metabolite. The genetic modification can be to a gene or genes encoding an enzyme that is operative in a metabolic pathway, such as an anabolic biosynthetic pathway or catabolic utilization pathway. Preferably, the host cell has all operative genes encoding a given biocatalytic activity deleted or inactivated in order to ensure removal of the biocatalytic activity. In a particular embodiment, the Pseudomonad is a *Pseudomonas fluorescens* cell.

[0113] One or more than one metabolic activity may be selected for knock-out or replacement. In the case of native auxotrophy(ies), additional metabolic knockouts or replacements can be provided. Where multiple activities are selected, the auxotrophy-restoring selection markers can be of a biosynthetic-type (anabolic), of a utilization-type (catabolic), or may be chosen from both types. For example, one or more than one activity in a given biosynthetic pathway for the selected compound may be knocked-out; or more than one activity, each from different biosynthetic pathways, may be knocked-out. The corresponding activity or activities are then provided by at least one recombinant vector which, upon transformation into the cell, restores prototrophy to the cell.

[0114] Compounds and molecules whose biosynthesis or utilization can be targeted to produce auxotrophic host cells include: lipids, including, for example, fatty acids; mono- and disaccharides and substituted derivatives thereof, including, for example, glucose, fructose, sucrose, glucose-6-phosphate, and glucuronic acid, as well as Entner-Doudoroff and Pentose Phosphate pathway intermediates and products; nucleosides, nucleotides, dinucleotides, including, for example, ATP, dCTP, FMN, FAD, NAD, NADP, nitrogenous bases, including, for example, pyridines, purines, pyrimidines, pterins, and hydro-, dehydro-, and/or substituted nitrogenous base derivatives, such as cofactors, for example, biotin, cobamamide, riboflavine, thiamine; organic acids and glycolysis and citric acid cycle intermediates and products, including, for example, hydroxyacids and amino acids; storage carbohydrates and storage poly(hydroxyalkanoate) polymers, including, for example, cellulose, starch, amylose, amylopectin, glycogen, poly-hydroxybutyrate, and polylactate.

[0115] In one embodiment, the biocatalytic activity(ies) knocked out to produce the auxotrophic host cell is selected from the group consisting of: the lipids; the nucleosides, nucleotides, dinucleotides, nitrogenous bases, and nitrogenous base derivatives; and the organic acids and glycolysis and citric acid cycle intermediates and products. Preferably, the biocatalytic activity(ies) knocked out is selected from the group consisting of: the nucleosides, nucleotides, dinucleotides, nitrogenous bases, and nitrogenous base derivatives; and the organic acids and glycolysis and citric acid cycle intermediates and products. More preferably, the biocatalytic activity(ies) knocked out is selected from the group consisting of: the pyrimidine nucleosides, nucleotides, dinucleotides, nitrogenous bases, and nitrogenous base derivatives; and the amino acids.

[0116] A given transgenic host cell may use one or more than one selection marker or selection marker system. For example, one or more biosynthesis selection marker(s) or selection marker system(s) according to the present invention may be used together with each other, and/or may be used in combination with a utilization-type selection marker or selection marker system according to the present invention. In any one of these prototrophy-enabling embodiments, the host cell may also contain one or more non-auxotrophic selection marker(s) or selection marker system(s). Examples of non-auxotrophic selection marker(s) and system(s) include, for example: toxin-resistance marker genes such as antibiotic-resistance genes that encode an enzymatic activity that degrades an antibiotic; toxin-resistant marker genes, such as, for example, imidazolinone-resistant mutants of acetolactate synthase ("ALS;" EC 2.2.1.6) in which mutation(s) are expressed that make the enzyme insensitive to toxin-inhibition exhibited by versions of the enzyme that do not contain such mutation(s). The compound(s) may exert this effect directly; or the compound(s) may exert this effect indirectly, for example, as a result of metabolic action of the cell that converts the compound(s) into toxin form or as a result of combination of the compound(s) with at least one further compound(s).

[0117] Bacterial-host-operative genes encoding such marker enzymes can be obtained from the bacterial host cell strain chosen for construction of the knock-out cell, from other bacteria, or from other organisms, and may be used in native form or modified (e.g., mutated or sequence recombined) form. For example, a DNA coding sequence for an enzyme exhibiting the knocked out biocatalytic activity may be obtained from one or more organisms and then operatively attached to DNA regulatory elements operative within the host cell. In specific, all of the chosen host's intracellular genes that encode a selected enzymatic activity are knocked-out; the bacterial knock-out host is then transformed with a vector containing at least one operative copy of a native or non-native gene encoding an enzyme exhibiting the activity lost by the bacterial knockout.

[0118] Bacterial and other genes encoding such enzymes can be selected and obtained through various resources available to one of ordinary skill in the art. These include the nucleotide sequences of enzyme coding sequences and species-operative DNA regulatory elements. Useful on-line InterNet resources include, e.g.,: (1) the ExPASy proteomics facility (see the ENZYME and BIOCHEMICAL PATHWAYS MAPS features) of the Swiss Institute of Bioinformatics (Bâatiment Ecole de Pharmacie, Room 3041; Universitéde Lausanne; 1015 Lausanne-Dorigny; Switzerland) available at, e.g., http://us.expasy.org/; and (2) the GenBank facility and other Entrez resources (see the PUBMED, PROTEIN, NUCLEOTIDE, STRUCTURE, GENOME, et al. features) offered by the National Center for Biotechnology Information (NCBI, National Library of Medicine, National Institutes of Health, U.S. Dept. of Health & Human Services; Building 38A; Bethesda, Md., USA) and available at http://www.ncbi.nlm.nih.gov/entrez/guery.fcgi.

[0119] The selected coding sequence may be modified by altering the genetic code thereof to match that employed by the bacterial host cell, and the codon sequence thereof may be enhanced to better approximate that employed by the host. Genetic code selection and codon frequency enhancement may be performed according to any of the various methods known to one of ordinary skill in the art, e.g., oligonucleotide-directed mutagenesis. Useful on-line InterNet resources to assist in this process include, e.g.: (1) the Codon Usage Database of the Kazusa DNA Research Institute (2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818 Japan) and available at http://www.kazusa.or.jp/codon/; and (2) the Genetic Codes tables available from the NCBI Taxonomy database at http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi?mode=c. For example, *Pseudomonas* species are reported as utilizing Genetic Code Translation Table 11 of the NCBI Taxonomy site, and at the Kazusa site as exhibiting the codon usage frequency of the table shown at http://www.kazusa.or.jp/codon/cgibin/.

[0120] In a particular embodiment, *Pseudomonas fluorescens* can be used as the host cell. In one embodiment, *Pseudomonas fluorescens* provides at least one auxotrophic selection marker gene. In an alternative embodiment, *Pseudomonas fluorescens* provides all auxotrophic selection marker genes. In a particular embodiment, *Pseudomonas fluorescens* can both be the host cell and provide at least one, and preferably all, auxotrophic selection marker genes.

[0121] Biosynthetic Nucleoside and Nitrogenous Base Selection Markers

[0122] In one embodiment, a biosynthetic enzyme involved in anabolic metabolism can be chosen as the auxotrophic selection marker. In particular, the biosynthetic enzyme can be selected from those involved in biosynthesis of the nucleosides, nucleotides, dinucleotides, nitrogenous bases, and nitrogenous base derivatives.

[0123] In a particular embodiment at least one purine-type biosynthetic enzyme can be chosen as an auxotrophic selection marker. Such purine biosynthetic enzymes include, for example, adenine phosphoribosyltransferases, adenylosuccinate lyases, adenylosuccinate synthases, GMP synthases, IMP cyclohydrolases, IMP dehydrogenases, phosphoribosylamine-glycine ligases, phosphoribosyl-aminoimidazolecarboxamide formyltransferases, phosphoribosylaminoimidazole carboxylases, phosphoribosyl aminoimidazolesuccinocarboxamide synthases, phosphoribosyl-formylglycinamidine cyclo ligases, phosphoribosyl-formylglycinamidine synthases, phosphoribosyl-glycinamide formyltransferases, ribose-phosphate diphosphokinases, and ribose-5-phosphate-ammonia ligases.

[0124] In another particular embodiment, a pyrimidine-type biosynthetic enzyme can be chosen as an auxotrophic

selection marker. Such pyrimidine-type biosynthetic include enzymes involved in biosynthesis of UMP, such as carbamate kinase (EC 2.7.2.2), carbamoyl-phosphate synthase (EC 6.3.5.5), aspartate carbamoyltransferase (EC 2.1.3.2), dihydroorotase (EC 3.5.2.3), dihydroorotate dehydrogenase (EC 1.3.3.1), orotate phosphoribosyltransferase ("OPRT;" EC 2.4.2.10), and orotidine-5'-phosphate decarboxylase ("ODCase;" EC 4.1.1.23).

[0125] Examples of genes encoding pyrimidine-type biosynthetic enzymes are well known. In the case of bacterial synthesis of UMP, examples of useful genes include: arcC genes, encoding carbamate kinases; carA and carB genes, collectively encoding carbamoyl-phosphate synthases; pyrB genes, encoding aspartate carbamoytransferases; pyrC genes, encoding dihydroorotases; pyrD genes, singly or collectively encoding dihydroorotate dehydrogenases; pyrE genes encoding orotate phosphoribosyltransferases; and pyrF genes, encoding orotidine-5'-phosphate decarboxylases.

[0126] In a particular embodiment, an expression system according to the present invention will utilize a pyrF auxotrophic selection marker gene. pyrF genes encode ODCase, an enzyme required for the bacterial pyrimidine nucleotide biosynthesis pathway, by which the cell performs de novo synthesis of pyrimidine nucleotides proper (UTP, CTP), as well as pyrimidine deoxynucleotides (dTTP, dCTP). The pathway's initial reactants are ATP, an amino group source (i.e. ammonium ion or L-glutamine), and a carboxyl group source (i.e. carbon dioxide or bicarbonate ion); the pathway's ultimate product is dTTP, with dCTP, UTP, and CTP also being formed in the process. Specifically, the bacterial de novo pyrimidine nucleotide biosynthesis pathway begins with the formation of carbamoyl phosphate. Carbamoyl phosphate is synthesized either: (a) by action of carbamate kinase (EC 2.7.2.2), encoded by the arcC gene; or, more commonly, (b) by action of the glutamine-hydrolyzing, carbamoyl-phosphate synthase (EC 6.3.5.5), whose small and large subunits are encoded by the carA and carB genes, respectively. Carbamoyl phosphate is then converted to UDP by the following six-step route: 1) conversion of carbamoyl phosphate to N-carbamoyl-L-aspartate, by aspartate carbamoyltransferase (EC 2.1.3.2), encoded by pyrB; then 2) conversion thereof to (S)-dihydroorotate, by dihydroorotase (EC 3.5.2.3), encoded by pyrC; then 3) conversion thereof to orotate, by dihydroorotate dehydrogenase (EC 1.3.3.1), encoded by pyrD gene(s); then 4) conversion thereof to orotidine-5'-monophosphate ("OMP"), by orotate phosphoribosyltransferase ("OPRT;" EC 2.4.2.10), encoded by pyrE; and then 5) conversion thereof to uridine-5'-monophosphate ("UMP"), by orotidine-5'-phosphate decarboxylase ("ODCase;" EC 4.1.1.23), encoded by pyrF. The UMP is then utilized by a variety of pathways for synthesis of pyrimidine nucleotides (UTP, CTP, dTTP, dCTP), nucleic acids, nucleoproteins, and other cellular metabolites.

[0127] In bacteria in which one or more of the carA, carB, or pyrB-pyrF genes has become inactivated or lost, or mutated to encode a non-functional enzyme, the cell can still thrive if uracil is added to the medium, provided that the cell contains a functioning uracil salvage pathway. Most bacteria contain a native uracil salvage pathway, including the Pseudomonads and related species. In a uracil salvage pathway, the cell imports and converts exogenous uracil into UMP, to synthesize the required pyrimidine nucleotides. In this, uracil is reacted with 5-phosphoribosyl-1-pyrophosphate to form UMP, by the action of either uracil phosphoribosyltransferase (EC 2.4.2.9), encoded by the upp gene, or by the bifunctional, pyrimidine operon regulatory protein ("pyrR bifunctional protein"), encoded by pyrR. The resulting UMP is then converted to UDP, and then the subsequent pyrimidine nucleotides, as described above.

[0128] Consequently, a pyrF(−) Pseudomonad or related cell can be maintained on uracil-containing medium. After a pyrF gene-containing DNA construct is transfected into the pyrF(−) cell and expressed to form a functioning ODCase enzyme, the resulting combined pyrF(+) plasmid-host cell system can be maintained in a medium lacking uracil.

[0129] The coding sequence of the pyrF gene for use in a Pseudomonad or related host cell can be provided by any gene encoding an orotidine-5'-phosphate decarboxylase enzyme ("ODCase"), provided that the coding sequence can be transcribed, translated, and otherwise processed by the selected Pseudomonad or related host cell to form a functioning ODCase. The pyrF coding sequence may be a native sequence, or it may be an engineered sequence resulting from, for example, application of one or more sequence-altering, sequence-combining, and/or sequence-generating techniques known in the art. Before use as part of a pyrF selection marker gene, the selected coding sequence may first be improved or optimized in accordance with the genetic code and/or the codon usage frequency of a selected Pseudomonad or related host cell. Expressible coding sequences will be operatively attached to a transcription promoter capable of functioning in the chosen host cell, as well as all other required transcription and translation regulatory elements. A native coding sequence for a pyrF gene as described above may be obtained from a bacterium or from any other organism, provided that it meets the above-described requirements.

[0130] In one embodiment, the pyrF coding sequence is isolated from the Pseudomonad or related host cell in which it is intended to be used as a selection marker. The entire pyrF gene (including the coding sequence and surrounding regulatory regions) can be isolated there from. In a particular embodiment, a bacterium providing the pyrF gene or coding sequence will be selected from the group consisting of a member of the order Pseudomonadales, a member of the suborder Pseudomonadineae, a member of the family Pseudomonadaceae, a member of the tribe Pseudomonadeae, a member of the genus *Pseudomonas,* and a member of the *Pseudomonas fluorescens* species group (i.e. the "fluorescent pseudomonads"). In a particular embodiment, the bacterium will belong to the species, *Pseudomonas fluorescens.*

[0131] In a particular embodiment, the pyrF gene contains the nucleic acid sequence of SEQ ID NO. 1 (Table 2), or a variant thereof. Alternatively, the ODCase encoded by the pyrF gene contains the amino acid sequence of SEQ ID NO. 2 (Table 3), a variant thereof, or a variant having a codon sequence redundant therewith, in accordance with the genetic code used by a given host cell according to the present invention.

[0132] Alternatively, the pyrF gene contains a nucleic acid sequence encoding an ODCase enzyme selected from the group consisting of a nucleic acid sequence at least 70%, 75%, 80%, 85%, 88%, 90%, and 95% homologous to SEQ

ID No. 1. Likewise, the pyrF gene encodes an ODCase selected from the group consisting of an amino acid sequence at least 70%, 75%, 80%, 85%, 88%, 90%, and 95% homologous to SEQ ID No. 2.

[0133] In another embodiment, the pyrF gene can contain a coding sequence having a nucleotide sequence at least 90%, 93%, 95%, 96%, 97%, 98% or 99% homologous to the nucleotide sequence of nucleotides 974-1669 of SEQ ID NO: 1.

[0134] In a particular embodiment, the pyrF gene can contain a coding sequence having a codon sequence that hybridizes to the anti-codon sequence of SEQ ID NO:3 (Table 4), when hybridization has been performed under highly stringent hybridization conditions, or can have a codon sequence redundant therewith. In a particularly particular embodiment, the pyrF gene will contain the nucleotide sequence of SEQ ID No. 3

TABLE 2

PSEUDOMONAS FLUORESCENS PYRF NUCLEIC ACID SEQUENCE

gatcagttgcggagccttggggtcatcccccagtttctgacgcaggcgcgacaccagcaagtcgatgctgcggtcga SEQ ID NO. 1

aagcctcgatggaacgcccacgggccgcgtccagcagctgttcgcggctcagcacacgccgcgggcgttcgataaac

acccacaacaaacgaaactcggcgttggacagcggcaccaccaggccgtcatcggccaccagctggcgcagtacgct

gttcaggcgccaagtgtcgaaacggatattggcccgctgttcggtgcggtcatcacgcacccggcgcaggatggtct

ggatacgcgcgaccagttcccggggttcgaacggcttggacatatagtcgtctgcccccagttccaggccgatgatg

cggtcggtgggttcgcagcgggcggtgagcatcaggatcggaatgtccgattcggcgcgcagccagcggcacaatgt

cagcccgtcttcgcccggcagcatcaggtcgagcaccaccacatcgaaggtctccgcttgcatggcctggcgcatgg

cgatgccgtcggtgacgcctgaggcgagaatattgaagcgtgccaggtagtcgatcagcagttcgcggatcggcacg

tcgtcgtcgacaatcagcgcgcgggtgttccagcgcttgtcttcggcgatcaccgcgtcttttggcgcttcgtttac

agggtcgcaaggggtatgcatagcgaggtcatctgcctggttgtggctgtcagcataggcgcccagttccagggctg

gaagtgctgggcgggcggtcatgtgcgcgaggctagccgggcggcgtattgggggcgtgtcgtgaatgtatcgggct

tgaaacaattgccttgaatcgccggtattgggcgcttgatcggtatttaccgatcatcggatcccgcaacggcgctg

cttgcgctacaatccgcgccgatttcgacttgcctgagagcccattccaatgtccgtctgccagactcctatcatcg

tcgccctggattaccccacccgtgacgccgcactgaagctggctgaccagttggaccccaagctttgccgggtcaag

gtcggcaaggaattgttcaccagttgcgcggcggaaatcgtcggcaccctgcgggacaaaggcttcgaagtgttcct

cgacctcaaattccatgacatccccaacaccacggcgatggccgtcaaagccgcggccgagatgggcgtgtggatgg

tcaatgtgcactgctccggtggcctgcgcatgatgagcgcctgccgcgaagtgctggaacagcgcagcggccccaaa

ccgttgttgatcggcgtgaccgtgctcaccagcatggagcgcgaagacctggcgggcattggcctggatatcgagcc

gcaggtgcaagtgttgcgcctggcagccctggcgcagaaagccggcctcgacggcctggtgtgctcagccctggaag

cccaggccctgaaaaacgcacatccgtcgctgcaactggtgacaccgggtatccgtcctaccggcagcgcccaggat

gaccagcgccgtatcctgaccccgcgccaggccctggatgcgggctctgactacctggtgatcggccggccgatcag

ccaggcggcggatcctgcaaaagcgttggcagcggtcgtcgccgagatcgcctgatttttagagtgagcaaaaaatg

tgggagctggcttgcctgcgatagtatcaactcggtatcacttagaaaccgagttgcttgcatcgcaggcaagccag

ctcccacatttgtttttgtggtgtgtcagctgactttgagcaccaacttcccgaagttctcgccgttgaacagcttc

atcagcgtttccgggaatgtctccagcccttcgacaatatcttccttgctcttgagcttgccctgggccatccagcc

ggccatttcctgacccgccgccgcgaagttcgccgcgtggtccatcaccacaaagccttccatacgcgcacggttga

ccagcaatgacaggtagttcgccgggcctttgaccgcttccttgttgttgtactggctgattgcaccgcaaatcacc

acgcgggctttgagcgccaggcggctgagcaccgcgtcgagaatatcgccgccgacgttatcgaaatacacgtccac

gcctttggggcactcgcgcttgagggcggcgggcacgtcttcgcttttgtagtcgatggcggcgtcgaagcccagct

catcgaccaggaacttgcacttctcggcgccaccggcgatccccactacgcgacagcctttgagcttagcgatctgc

ccggcgatgctgcccacggcaccggcggcgccggagatcaccacggtgtcaccggctttcggtgcgccggtctccag

TABLE 2-continued

PSEUDOMONAS FLUORESCENS PYRF NUCLEIC ACID SEQUENCE

cagagcaaagtaggccgtcatgccggtcatgcccagggcggacaggtagcggggcaggggcgccagcttggggtcca

ccttatagaaaccacggggctcgccaaggaagtaatcctgcacgcccagtgcaccgttcacgtagtcccccaccgcg

aagttcggatggttcgaggcaagcaccttgcctacgcccagggcgcgcatcacttcgccgatgcctaccggtgggat

gtaggacttgccttcattcatccagccacgca

[0135]

TABLE 3

PSEUDOMONAS FLUORESCENS ODCASE AMINO ACID SEQUENCE

Met Ser Val Cys Gln Thr Pro Ile Ile Val Ala Leu Asp Tyr Pro Thr Arg Asp SEQ ID NO. 2

Ala Ala Leu Lys Leu Ala Asp Gln Leu Asp Pro Lys Leu Cys Arg Val Lys Val

Gly Lys Glu Leu Phe Thr Ser Cys Ala Ala Glu Ile Val Gly Thr Leu Arg Asp

Lys Gly Phe Glu Val Phe Leu Asp Leu Lys Phe His Asp Ile Pro Asn Thr Thr

Ala Met Ala Val Lys Ala Ala Ala Glu Met Gly Val Trp Met Val Asn Val His

Cys Ser Gly Gly Leu Arg Met Met Ser Ala Cys Arg Glu Val Leu Glu Gln Arg

Ser Gly Pro Lys Pro Leu Leu Ile Gly Val Thr Val Leu Thr Ser Met Glu Arg

Glu Asp Leu Ala Gly Ile Gly Leu Asp Ile Glu Pro Gln Val Gln Val Leu Arg

Leu Ala Ala Leu Ala Gln Lys Ala Gly Leu Asp Gly Leu Val Cys Ser Ala Leu

Glu Ala Gln Ala Leu Lys Asn Ala His Pro Ser Leu Gln Leu Val Thr Pro Gly

Ile Arg Pro Thr Gly Ser Ala Gln Asp Asp Gln Arg Arg Ile Leu Thr Pro Arg

Gln Ala Leu Asp Ala Gly Ser Asp Tyr Leu Val Ile Gly Arg Pro Ile Ser Gln

Ala Ala Asp Pro Ala Lys Ala Leu Ala Ala Val Val Ala Glu Ile Ala

[0136]

TABLE 4

PSEUDOMONAS FLUORESCENS PYRF NUCLEIC ACID SEQUENCE

atgtccgtctgccagactcctatcatcgtcgccctggattaccccacccgtgacgccgcactgaag SEQ. ID No. 3

ctggctgaccagttggaccccaagctttgccgggtcaaggtcggcaaggaattgttcaccagttgc

gcggcggaaatcgtcggcaccctgcgggacaaaggcttcgaagtgttcctcgacctcaaattccat

gacatccccaacaccacggcgatggccgtcaaagccgcggccgagatgggcgtgtggatggtcaat

gtgcactgctccggtggcctgcgcatgatgagcgcctgccgcgaagtgctggaacagcgcagcggc

cccaaaccgttgttgatcggcgtgaccgtgctcaccagcatggagcgcgaagacctggcgggcatt

ggcctggatatcgagccgcaggtgcaagtgttgcgcctggcagccctggcgcagaaagccggcctc

gacggcctggtgtgctcagccctggaagcccaggccctgaaaaacgcacatccgtcgctgcaactg

gtgacaccgggtatccgtcctaccggcagcgcccaggatgaccagcgccgtatcctgaccccgcgc

TABLE 4-continued

PSEUDOMONAS FLUORESCENS PYRF NUCLEIC ACID SEQUENCE

caggccctggatgcgggctctgactacctggtgatcggccggccgatcagccaggcggcggatcct

gcaaaagcgttggcagcggtcgtcgccgagatcgcc

[0137] In an alternate embodiment, an expression system according to the present invention will utilize a thyA auxotrophic selection marker gene. thyA genes encode thymidylate synthase (EC 2.1.1.45), an enzyme required for the bacterial pyrimidine nucleotide biosynthesis pathway. Since DNA contains thymine (5-methyluracil) as a major base instead of uracil, the synthesis of thymidine monophospate (dTMP or thymidylate) is essential to provide dTTP (thymidine triphosphate) needed for DNA replication together with dATP, dGTP, and dCTP. Methylation of dUMP by thymidylate synthase utilizing 5,10-methylenetetrahydrofolate as the source of the methyl group generates thymidylate. Thymidylate synthesis can be interrupted, and consequently the synthesis of DNA arrested, by the removal, inhibition, or disruption of thymidylate synthase.

[0138] In bacteria in which the thyA gene has become inactivated or lost, or mutated to encode a non-functional enzyme, the cell can still thrive if exogenous thymidine is added to the medium.

[0139] In *Pseudomonas fluorescens,* the addition of an *E.coli* tdk gene, encoding thymidine kinase, is required for survival on exogenous thymidine. Therefore, prior to selection, a plasmid comprising a tdk gene can be used to transform thyA(−) *P. fluorescens* host cells, generating a thyA(−)/ptdk cell, allowing survival on a thymidine containing medium. Alternatively, a tdk gene producing a functional thymidylate synthase enzyme capable of utilizing exogenous thymidine in *Pseudomonas fluorescens* can be inserted into the genome, producing a thyA(−)/tdk(+) host cell. After a thyA gene-containing DNA construct is transfected into the thyA(−)/ptdk cell and expressed to form a functioning thymidylate synthase enzyme, the resulting combined thyA(+) plasmid-host cell system can be maintained in a medium lacking thymidine.

[0140] The coding sequence of the thyA gene for use in a Pseudomonad or related host cell can be provided by any gene encoding a thymidylate synthase enzyme ("TS"), provided that the coding sequence can be transcribed, translated, and otherwise processed by the selected Pseudomonad or related host cell to form a functioning TS. The thyA coding sequence may be a native sequence, or it may be an engineered sequence resulting from, for example, application of one or more sequence-altering, sequence-combining, and/or sequence-generating techniques known in the art. Before use as part of a thyA selection marker gene, the selected coding sequence may first be improved or optimized in accordance with the genetic code and/or the codon usage frequency of a selected Pseudomonad or related host cell. Expressible coding sequences will be operatively attached to a transcription promoter capable of functioning in the chosen host cell, as well as all other required transcription and translation regulatory elements. A native coding sequence for a thyA gene as described above may be obtained from a bacterium or from any other organism, provided that it meets the above-described requirements.

[0141] In one embodiment, the thyA coding sequence is isolated from the Pseudomonad or related host cell in which it is intended to be used as a selection marker. The entire thyA gene (including the coding sequence and surrounding regulatory regions) can be isolated there from. In a particular embodiment, a bacterium providing the thyA gene or coding sequence will be selected from the group consisting of a member of the order Pseudomonadales, a member of the suborder Pseudomonadineae, a member of the family Pseudomonadaceae, a member of the tribe Pseudomonadeae, a member of the genus *Pseudomonas,* and a member of the *Pseudomonas fluorescens* species group (i.e. the "fluorescent pseudomonads"). In a particular embodiment, the bacterium will belong to the species, *Pseudomonas fluorescens.*

[0142] In a particular embodiment, the thyA gene contains the nucleic acid sequence of SEQ ID NO. 4 (Table 5). Alternatively, the TS encoded by the thyA gene contains the amino acid sequence of SEQ ID NO. 5 (Table 6), a variant thereof, or a variant having a codon sequence redundant therewith, in accordance with the genetic code used by a given host cell according to the present invention.

TABLE 5

PSEUDOMONAS FLUORESCENS THYA NUCLEIC ACID SEQUENCE

atgaagcaatatctcgaactactgaacgacgtcgtgaccaatggattgaccaagggcgatcgcac SEQ ID NO. 4

cggcaccggcaccaaagccgtatttgcccgtcagtatcggcataacttggccgacggcttcccgc

tgctgaccaccaagaagcttcatttcaaaagtatcgccaacgagttgatctggatgttgagcggc

aacaccaacatcaagtggctcaacgaaaatggcgtgaaaatctgggacgagtgggccaccgaaga

cggcgacctgggcccggtgtacggcgagcaatggaccgcctggccgaccaaggacggcggcaaga

tcaaccagatcgactacatggtccacaccctcaaaaccaaccccaacagccgccgcatcctgttt

TABLE 5-continued

PSEUDOMONAS FLUORESCENS THYA NUCLEIC ACID SEQUENCE

catggctggaacgtcgagtacctgccggacgaaaccaagagcccgcaggagaacgcgcgcaacgg

caagcaagccttgccgccgtgccatctgttgtaccaggcgttcgtgcatgacgggcatctgtcga

tgcagttgtatatccgcagctccgacgtcttcctcggcctgccgtacaacaccgccgcgttggcc

ttgctgactcacatgctggctcagcaatgcgacctgatccctcacgagatcatcgtcaccaccgg

cgacacccatgcttacagcaaccacatggaacagatccgcacccagctggcgcgtacgccgaaaa

agctgccggaactggtgatcaagcgtaaacctgcgtcgatctacgattacaagtttgaagacttt

gaaatcgttggctacgacgccgacccgagcatcaaggctgacgtggctatctga

[0143]

TABLE 6

PSEUDOMONAS FLUORESCENS TS AMINO ACID SEQUENCE

MKQYLELLNDVVTNGLTKGDRTGTGTKAVFARQYRHNLADGFPLLTTKKLHFKSIANELIWMLSG SEQ ID NO. 5

NTNIKWLNENGVKIWDEWATEDGDLGPVYGEQWTAWPTKDGGKINQIDYMVHTLKTNPNSRRILF

HGWNVEYLPDETKSPQENARNGKQALPPCHLLYQAFVHDGHLSMQLYIRSSDVFLGLPYNTAALA

LLTHMLAQQCDLIPHEIIVTTGDTHAYSNHMEQIRTQLARTPKKLPELVIKRKPASIYDYKFEDF

EIVGYDADPSIKADVAI

[0144] Biosynthetic Amino Acid Selection Markers

[0145] In an alternative embodiment, the biosynthetic enzyme involved in anabolic metabolism chosen as the auxotrophic selection marker can be selected from those involved in the biosynthesis of amino acids. In particular embodiments, the biosynthetic amino acid enzymes are selected from the group consisting of enzymes active in the biosynthesis of: the Glutamate Family (Glu; Gln, Pro, and Arg); the Aspartate Family (Asp; Asn, Met, Thr, Lys, and Ile); the Serine Family (Ser; Gly and Cys); the Pyruvate Family (Ala, Val, and Leu); the Aromatic Family (Trp, Phe, and Tyr); and the Histidine Family (His). Examples of genes and enzymes involved in these biosynthetic pathways include: the Glutamate Family member arg, gdh, gln, and, pro genes, including, for example, argA-argH, gdhA, glnA, proA, proC; the Aspartate Family member asd, asn, asp, dap, lys, met, and thr genes, including, for example, asnA, asnB, aspC, dapA, dapB, dapD-dapF, lysA, lysC, metA-metC, metE, metH, metL, thrA-thrC; the Serine Family member cys, gly, and ser genes, including, for example, cysE, cysK, glyA, serA-serC; the Aromatic Family member aro, phe, trp, and tyr genes, including, for example, aroA-aroH, aroK, aroL, trpAtrpE, tyrA, and tyrB; and the Histidine Family member his genes, including hisA-hisD, hisF-hisH.

[0146] In a further particular embodiment, the auxotrophic selection marker can be selected from enzymes involved in the biosynthesis of members of the Glutamate Family. Examples of useful Glutamate Family auxotrophic selection markers include the following, listed with representative examples of their encoding genes: argA, encoding N-acetylglutamate synthases, amino acid acetyltransferases; argB, encoding acetylglutamate kinases; argC, encoding N-acetylgammaglutamylphosphate reductases; argD, encoding acetylornithine delta-aminotransferases; argE, encoding acetylornithine deacetylases; argF and argI, encoding ornithine carbamoyltransferases; argG, encoding argininosuccinate synthetases; argH, encoding argininosuccinate lyases; gdhA, encoding glutamate dehydrogenases; glnA, encoding glutamine synthetases; proA, encoding gamma-glutamylphosphate reductases; proB, encoding gamma-glutamate kinases; and proC, encoding pyrroline-5-carboxylate reductases.

[0147] In one embodiment, an amino acid biosynthesis selection marker gene can be at least one member of the proline biosynthesis family, in particular proA, proB, or proC. In a particular embodiment, the proline biosynthesis selection marker gene can comprise a proC gene. proC genes encode an enzyme catalyzing the final step of the proline biosynthesis pathway. In bacteria, the proline (i.e. L-proline) biosynthesis pathway comprises a three-enzyme process, beginning with L-glutamic acid. The steps of this process are: 1) conversion of L-glutamic acid to L-glutamyl-5-phosphate, by glutamate-5-kinase ("GK;" EC 2.7.2.11), encoded by proB; then 2a) conversion thereof to L-glutamate-5-semialdehyde, by glutamate-5-semialdehyde dehydrogenase (EC 1.2.1.41), also known as glutamyl-5-phosphate reductase ("GPR"), encoded by proA, followed by 2b) spontaneous cyclization thereof to form .1-pyrroline-5-carboxylate; and then 3) conversion thereof to L-proline, by Δ^1-pyrroline-5-carboxylate reductase ("P5CR;" EC

1.5.1.2), encoded by proC. In most bacteria, proC encodes the P5CR subunit, with the active P5CR enzyme being a homo-multimer thereof.

[0148] In bacteria in which one or more of the proA, proB, or proC genes has become inactivated or lost, or mutated to encode a non-functional enzyme, the cell can still thrive if proline is added to the medium. Consequently, a proC(−) Pseudomonad or related cell can be maintained on a proline-containing medium. After a proC gene-containing DNA construct is transfected into the proC(−) cell and expressed to form a functioning P5CR enzyme, the resulting combined proC(+) plasmid-host cell system can be maintained in a medium lacking proline.

[0149] The coding sequence of the proC gene for use in a Pseudomonad or related host cell can be provided by any gene encoding an Δ^1-pyrroline-5-carboxylate reductase enzyme (P5CR), provided that the coding sequence can be transcribed, translated, and otherwise processed by the selected Pseudomonad or related host cell to form a functioning P5CR. The proC coding sequence may be a native sequence, or it may be an engineered sequence resulting from, for example, application of one or more sequence-altering, sequence-combining, and/or sequence-generating techniques known in the art. Before use as part of a proC selection marker gene, the selected coding sequence may first be improved or optimized in accordance with the genetic code and/or the codon usage frequency of a selected Pseudomonad or related host cell. Expressible coding sequences will be operatively attached to a transcription promoter capable of functioning in the chosen host cell, as well as all other required transcription and translation regulatory elements. A native coding sequence for a proC gene as described above may be obtained from a bacterium or from any other organism, provided that it meets the above-described requirements.

[0150] In one embodiment, the proC coding sequence is isolated from the Pseudomonad or related host cell in which it is intended to be used as a selection marker. The entire proC gene (including the coding sequence and surrounding regulatory regions) can be isolated therefrom. In a particular embodiment, a bacterium providing the proC gene or coding sequence will be selected from the group consisting of a member of the order Pseudomonadales, a member of the suborder Pseudomonadineae, a member of the family Pseudomonadaceae, a member of the tribe Pseudomonadeae, a member of the genus *Pseudomonas,* and a member of the *Pseudomonas fluorescens* species group (i.e. the "fluorescent pseudomonads"). In a particular embodiment, the bacterium will belong to the species, *Pseudomonas fluorescens.*

[0151] In a particular embodiment, the proC gene contains the nucleic acid sequence of SEQ ID NO. 6 (Table 7), or a variant thereof. Alternatively, the P5CR encoded by the proC gene contains the amino acid sequence of SEQ ID NO. 7 (Table 8), a variant thereof, or a variant having a codon sequence redundant therewith, in accordance with the genetic code used by a given host cell according to the present invention.

[0152] Alternatively, the proC gene contains a nucleic acid sequence encoding an P5CR enzyme that is at least 70%, 75%, 80%, 85%, 88%, 90%, and 95% homologous to SEQ ID No. 6. Likewise, the proC gene encodes an ODCase that is at least 70%, 75%, 80%, 85%, 88%, 90%, and 95% homologous to SEQ ID No. 7.

[0153] In another embodiment, the proC gene can contain a coding sequence at least 90%, 93%, 95%, 96%, 97%, 98% or 99% homologous to the nucleotide sequence of SEQ. ID NO. 8 (Table 9).

[0154] In a particular embodiment, the proC gene can contain a coding sequence having a codon sequence that hybridizes to the anti-codon sequence of SEQ ID NO. 8, when hybridization has been performed under stringent hybridization conditions, or can have a codon sequence redundant therewith. In a particularly particular embodiment, the proC gene will contain the nucleotide sequence of SEQ ID NO. 8.

TABLE 7

PSEUDOMONAS FLUORESCENS PROC NUCLEIC ACID SEQUENCE

gcccttgagttggcacttcatcggccccattcaatcgaacaagactcgtgccatcgccgagcacttcgcttgg SEQ ID NO. 6

gtgcactccgtggaccgcctgaaaatcgcacaacgcctgtccgaacaacgcccggccgacctgccgccgctca

atatctgcatccaggtcaatgtcagtggcgaagccagcaagtccggctgcacgcccgctgacctgccggccct

ggccacagcgatcagcgccctgccgcgcttgaagctgcggggcttgatggcgattcccgagccgacgcaagac

cgggcggagcaggatgcggcgttcgccacggtgcgcgacttgcaagccagcttgaacctggcgctggacacac

tttccatgggcatgagccacgaccttgagtcggccattgcccaaggcgccacctgggtgcggatcggtaccgc

cctgtttggcgcccgcgactacggccagccgtgaaatggctgacatccctcgaaataaggacctgtcatgagc

aacacgcgtattgcctttatcggcgccggtaacatggcggccagcctgatcggtggcctgcgggccaagggcc

tggacgccgagcagatccgcgccagcgaccccggtgccgaaacccgcgagcgcgtcagagccgaacacggtat

ccagaccttcgccgataacgccgaggccatccacggcgtcgatgtgatcgtgctggcggtcaagccccaggcc

atgaaggccgtgtgcgagagcctgagcccgagcctgcaaccccatcaactggtggtgtcgattgccgctggca

tcacctgcgccagcatgaccaactggctcggtgcccagcccattgtgcgctgcatgcccaacaccccggcgct

TABLE 7-continued

PSEUDOMONAS FLUORESCENS PROC NUCLEIC ACID SEQUENCE

gctgcgccagggcgtcagcggtttgtatgccactggcgaagtcaccgcgcagcaacgtgaccaggcccaggaa

ctgctgtctgcggtgggcatcgccgtgtggctggagcaggaacagcaactggatgcggtcaccgccgtctccg

gcagcggcccggcttacttcttcctgttgatcgaggccatgacggccgcaggcgtcaagctgggcctgcccca

cgacgtggccgagcaactggcggaacaaaccgccctgggcgccgccaagatggcggtcggcagcgaggtggat

gccgccgaactgcgccgtcgcgtcacctcgccaggtggtaccacacaagcggctattgagtcgttccaggccg

ggggctttgaagccctggtggaaacagcactgggtgccgccgcacatcgttcagccgagatggctgagcaact

gggcaaatagtcgtcccttaccaaggtaatcaaacatgctcggaatcaatgacgctgccattttcatcatcca

gaccctgggcagcctgtacctgctgatcgtactgatgcgctttatcctgcaactggtgcgtgcgaacttctac

aacccgctgtgccagttcgtggtgaaggccacccaaccgctgctcaagccgctgcgccgggtgatcccgagcc

tgttcggcctggacatgtcgtcgctggtgctggcgctgttgctgcagattttgctgttcgtggtgatcctgat

gctcaatggataccaggccttcaccgtgctgctgttgccatggggcctgatcgggattttctcgctgttcctg

aagatcattttctggtcgatgatcatcagcgtgatcctgtcctgggtcgcaccgggtagccgtagcccgggtg

ccgaattggtggctcagatcaccgagccggtgctggcacccttccgtcgcctgattccgaacctgggtggcct

ggatatctcgccgatcttcgcgtttatc

[0155]

TABLE 8

PSEUDOMONAS FLUORESCENS P5CR AMINO ACID SEQUENCE

Met Ser Asn Thr Arg Ile Ala Phe Ile Gly Ala Gly Asn Met Ala Ala Ser Leu SEQ ID NO. 7

Ile Gly Gly Leu Arg Ala Lys Gly Leu Asp Ala Glu Gln Ile Arg Ala Ser Asp

Pro Gly Ala Glu Thr Arg Glu Arg Val Arg Ala Glu His Gly Ile Gln Thr Phe

Ala Asp Asn Ala Glu Ala Ile His Gly Val Asp Val Ile Val Leu Ala Val Lys

Pro Gln Ala Met Lys Ala Val Cys Glu Ser Leu Ser Pro Ser Leu Gln Pro His

Gln Leu Val Val Ser Ile Ala Ala Gly Ile Thr Cys Ala Ser Met Thr Asn Trp

Leu Gly Ala Gln Pro Ile Val Arg Cys Met Pro Asn Thr Pro Ala Leu Leu Arg

Gln Gly Val Ser Gly Leu Tyr Ala Thr Gly Glu Val Thr Ala Gln Gln Arg Asp

Gln Ala Gln Glu Leu Leu Ser Ala Val Gly Ile Ala Val Trp Leu Glu Gln Glu

Gln Gln Leu Asp Ala Val Thr Ala Val Ser Gly Ser Gly Pro Ala Tyr Phe Phe

Leu Leu Ile Glu Ala Met Thr Ala Ala Gly Val Lys Leu Gly Leu Pro His Asp

Val Ala Glu Gln Leu Ala Glu Gln Thr Ala Leu Gly Ala Ala Lys Met Ala Val

Gly Ser Glu Val Asp Ala Ala Glu Leu Arg Arg Arg Val Thr Ser Pro Gly Gly

Thr Thr Gln Ala Ala Ile Glu Ser Phe Gln Ala Gly Gly Phe Glu Ala Leu Val

Glu Thr Ala Leu Gly Ala Ala Ala His Arg Ser Ala Glu Met Ala Glu Gln Leu

Gly Lys

[0156]

TABLE 9

PSEUDOMONAS FLUORESCENS PROC NUCLEIC ACID SEQUENCE

atgagcaacacgcgtattgcctttatcggcgccggtaacatggcggccagcctgatcggtggc SEQ ID NO. 8

ctgcgggccaagggcctggacgccgagcagatccgcgccagcgaccccggtgccgaaacccgc

gagcgcgtcagagccgaacacggtatccagaccttcgccgataacgccgaggccatccacggc

gtcgatgtgatcgtgctggcggtcaagccccaggccatgaaggccgtgtgcgagagcctgagc

ccgagcctgcaaccccatcaactggtggtgtcgattgccgctggcatcacctgcgccagcatg

accaactggctcggtgcccagcccattgtgcgctgcatgcccaacaccccggcgctgctgcgc

cagggcgtcagcggtttgtatgccactggcgaagtcaccgcgcagcaacgtgaccaggcccag

gaactgctgtctgcggtgggcatcgccgtgtggctggagcaggaacagcaactggatgcggtc

accgccgtctccggcagcggcccggcttacttcttcctgttgatcgaggccatgacggccgca

ggcgtcaagctgggcctgccccacgacgtggccgagcaactggcggaacaaaccgccctgggc

gccgccaagatggcggtcggcagcgaggtggatgccgccgaactgcgccgtcgcgtcacctcg

ccaggtggtaccacacaagcggctattgagtcgttccaggccgggggctttgaagccctggtg

gaaacagcactgggtgccgccgcacatcgttcagccgagatggctgagcaactgggcaaa

[0157] Utilization Selection Markers

[0158] In one embodiment, an enzyme involved in the catabolic utilization of metabolites can be chosen as the auxotrophic selection marker. In particular, the enzymes can be selected from those involved in the utilization of a carbon source. Examples of such enzymes include, for example, sucrases, lactases, maltases, starch catabolic enzymes, glycogen catabolic enzymes, cellulases, and poly(hydroxyalkanoate)depolymerases. If the bacterial host cell exhibits native catabolic activity of the selected type, it can be knocked-out before transformation with the prototrophy-restoring vector. Bacteria exhibiting native auxotrophy for these compounds can also be used in their native state for such transformation. In those embodiments in which a compound not importable or diffusible into the cell can be selected and supplied to the medium, the prototrophy restoring or prototrophy-enabling enzyme(s) can be secreted for use. In that case, the secreted enzyme(s) can degrade the compound extracellularly to produce smaller compounds, for example glucose, that are diffusible or importable into the cell, by selecting or designing the coding sequence of the enzyme(s) to include a coding sequence for a secretion signal peptide operative within the chosen host cell. In these embodiments, the prototrophy-restorative gene can be selected or be engineered to include a coding sequence for a secretion signal peptide operative within the chosen host cell to obtaining transport of the enzyme across the cytoplasmic membrane. In either of these embodiments, or those in which the selected compound is importable or diffusible into the cell, the cell will be grown in medium supplying no other carbon source apart from the selected compound.

[0159] In a carbon-source-utilization-based marker system, every prototrophy-restorative or prototrophy-enabling carbon-source utilization enzyme can be involved in utilization of only one carbon source. For example, two genes from the same catabolic pathway may be expressed together on one vector or may be co-expressed separately on different vectors in order to provide the prototrophy. Specific examples of such multi-gene carbon-source-utilization-based marker systems include, for example, the use of glycogen as the sole carbon source with transgenic expression of both a glycogen phosphorylase and an (alpha-1, 4)glucantransferase; and the use of starch as the sole carbon source with transgenic expression of both an alpha-amylase, and an alpha(1->6) glucosidase. However, the selected single- or multi-gene carbon-source marker system can be used simultaneously with other types of marker system(s) in the same host cell, provided that the only carbon source provided to the cell is the compound selected for use in the carbon-source catabolic selection marker system.

[0160] Other examples of useful enzymes for biochemical-utilization-type activities are well known in the art, and can include racemases and epimerases that are capable of converting a non-utilizable D-carbon source, supplied to the cell, to a nutritive L-carbon source. Examples of these systems include, for example: a D-acid or a D-acyl compound used with trangenic expression of the corresponding racemase; and lactate used with transgenically expressed lactate racemase.

[0161] Similarly, where an amino acid biosynthetic activity has been selected for use in the marker system, the auxotrophy may also be overcome by supplying the cell with both a non-utilizable R-amino acid and an R-amino acid racemase or epimerase (EC 5.1.1) that converts the R-amino acid into the corresponding L-amino acid for which the cell is auxotrophic.

[0162] Trait Stacking

[0163] A plurality of phenotypic changes can also be made to a host cell, before or after insertion of an auxotrophic

selection marker gene, for target gene expression, according to the present invention. For example, the cell can be genetically engineered, either simultaneously or sequentially, to exhibit a variety of enhancing phenotypic traits. This process is referred to as "trait stacking." A pryF deletion may be present as one such phenotypic trait. In such a strain, a pyrF gene, according to the present invention, can be used on a suicide vector as both a selectable marker and a counterselectable marker (in the presence of 5'-fluoro-orotic acid) in order to effect a cross-in/cross-out allele exchange of other desirable traits, Thus, a pyrF gene according to the present invention may be used in a process for "trait stacking" a host cell. In such a process, a suicide vector containing such a pyrF gene can be transformed into the host cell strain in a plurality of separate transformations; in each such procedure the re-establishment of the pyrF phenotype can be used to create, ad infinitum, subsequent genetically-enhancing phenotypic change. Thus, not only can the pyrF gene itself provide a trait, it can be used to obtain additional phenotypic traits in a process of trait-stacking.

[0164] In one embodiment, the present invention provides auxotrophic Pseudomonads and related bacteria that have been further genetically modified to induce additional auxotrophies. For example, a pyrF(−) auxotroph can be further modified to inactivate another biosynthetic enzyme present in an anabolic or catabolic pathway, such as through the inactivation of a proC gene or a thyA gene. In this way, multiple auxotrophies in the host cell can be produced.

[0165] In another embodiment, genetic alterations can be made to the host cell in order to improve the expression of recombinant polypeptides in the host cell. Further modifications can include genetic alterations that allow for a more efficient utilization of a particular carbon source, thereby optimizing the overall efficiency of the entire fermentation.

[0166] In one particular embodiment, auxotrophic host cells are further modified by the insertion of a lacI containing transgene into the host chromosome. Preferably, the lacI transgene, or derivate thereof, is other than part of a whole or truncated structural gene containing PlacI-lacI-lacZYA construct.

[0167] Modifications to Induce Auxotrophism

[0168] A Pseudomonad or related host cell selected for use in an expression system according to the present invention can be deficient in its ability to express any functional biocatalyst exhibiting the selected auxotrophic activity. For example, where an orotidine-5'-phosphate decarboxylase activity is selected, the host cell can be deficient in its ability to express a) any pyrF gene product (i.e. any functional ODCase enzyme), and b) any effective replacement therefore (i.e. any other biocatalyst having ODCase activity). In a one embodiment, the host cell will be made biocatalytically-deficient for the selected activity by altering its genomic gene(s) so that the cell cannot express, from its genome, a functional enzyme involved in the targeted auxotrophy (i.e. ODCase). In other words, the prototrophic cell (activity(+) cell) will become auxotrophic through the "knock-out" of a functional enzymatic encoding gene involved in the targeted prototrophic pathway (i.e. an activity(−) cell). This alteration can be done by altering the cell's genomic coding sequence(s) of the gene(s) encoding the selected activty(ies). In one embodiment, the coding sequence alteration(s) will be accomplished by introducing: insertion or deletion mutation(s) that change the coding sequence reading frame(s); substitution or inversion mutations that alter a sufficient number of codons; and/or deletion mutations that delete a sufficiently large group of contiguous codons there from capable of producing a non-functional enzyme.

[0169] In a one embodiment in which the host cell strain has also provided the auxotrophic gene(s) for use as selection marker(s) therein, preferably each of the selected gene's transcription promoter and/or transcription terminator element(s) can also be inactivated by introduction of mutation(s), including deletion mutations. For example, the transcription element inactivation can be optionally performed in addition to the coding sequence alteration(s) described above. In a one embodiment in which the host cell strain has also provided the auxotrophic selection marker gene(s), all of the selected gene(s)'s DNA can be deleted from the host cell genome.

[0170] Such knock-out strains can be prepared according to any of the various methods known in the art as effective. For example, homologous recombination vectors containing homologous targeted gene sequences 5' and 3' of the desired nucleic acid deletion sequence can be transformed into the host cell. Ideally, upon homologous recombination, a desired targeted enzymatic gene knock-out can be produced.

[0171] Specific examples of gene knock-out methodologies include, for example: Gene inactivation by insertion of a polynucleotide has been previously described. See, e.g., D L Roeder & A Collmer, *Marker-exchange mutagenesis of a pectate lyase isozyme gene in Erwinia chrysanthemi,* J Bacteriol. 164(1):51-56 (1985). Alternatively, transposon mutagenesis and selection for desired phenotype (such as the inability to metabolize benzoate or anthranilate) can be used to isolate bacterial strains in which target genes have been insertionally inactivated. See, e.g., K Nida & P P Cleary, *Insertional inactivation of streptolysin S expression in Streptococcus pyogenes,* J Bacteriol. 155(3):1156-61 (1983). Specific mutations or deletions in a particular gene can be constructed using cassette mutagenesis, for example, as described in J A Wells et al., *Cassette mutagenesis: an efficient method for generation of multiple mutations at defined sites,* Gene 34(2-3):315-23 (1985); whereby direct or random mutations are made in a selected portion of a gene, and then incorporated into the chromosomal copy of the gene by homologous recombination.

[0172] In one embodiment, both the organism from which the selection marker gene(s) is obtained and the host cell in which the selection marker gene(s) is utilized can be selected from a prokaryote. In a particular embodiment, both the organism from which the selection marker gene(s) is obtained and the host cell in which a selection marker gene(s) is utilized can be selected from a bacteria. In another embodiment, both the bacteria from which the selection marker gene(s) is obtained and the bacterial host cell in which a selection marker gene(s) is utilized, will be selected from the Proteobacteria. In still another embodiment, both the bacteria from which the selection marker gene(s) is obtained and the bacterial host cells in which a selection marker gene(s) is utilized, can be selected from the Pseudomonads and closely related bacteria or from a Subgroup thereof, as defined below.

[0173] In a particular embodiment, both the selection marker gene(s) source organism and the host cell can be

selected from the same species. Preferably, the species will be a prokaryote; more preferably a bacterium, still more preferably a Proteobacterium. In another particular embodiment, both the selection marker gene(s) source organism and the host cell can be selected from the same species in a genus selected from the Pseudomonads and closely related bacteria or from a Subgroup thereof, as defined below. In one embodiment, both the selection marker gene(s) source organism and the host cell can be selected from a species of the genus *Pseudomonas,* particularly the species *Pseudomonas fluorescens,* and preferably the species *Pseudomonas fluorescens* biotype A.

III. LacI Insertion

[0174] The present invention provides Pseudomonads and related cells that have been genetically modified to contain a chromosomally insert lacI transgene or derivative, other than as part of a whole or truncated PlacI-lacI-lacZYA operon. In one embodiment, the lacI insert provides stringent expression vector control through the expression of the LacI repressor protein which binds to the lacO sequence or derivative on the vector, and inhibits a Plac-Ptac family promoter on the vector. The result is reduced basal levels of recombinant polypeptide expression prior to induction.

[0175] In one embodiment, Pseudomonad host cells containing a chromosomal insertion of a native *E.coli* lacI gene, or lacI gene derivative such as $lacI^{Q}$ or $lacI^{Q\ 1,}$ are provided wherein the lacI insert is other than part of a whole or truncated, structural gene-containing PlacI-lacI-lacZYA construct. Other derivative lacI transgenes useful in the present invention include: lacI derivatives that have altered codon sequences different from a native lacI gene (for example, the native *E.coli* lacI gene contains a 'gtg' initiation codon, and this may be replaced by an alternative initiation codon effective for translation initiation in the selected expression host cell, e.g., 'atg'); lacI derivatives that encode LacI proteins having mutated amino acid sequences, including temperature-sensitive lacI mutants, such as that encoded by $lacI^{ts}$ (or "lacI(Ts)"), which respond to a shift in temperature in order to achieve target gene induction, e.g., a shift up to 42° C. (see, e.g., Bukrinsky et al., *Gene* 70:415-17 (1989); N Hasan & W Szybalski, *Gene* 163(1):35-40 (1995); H Adari et al., *DNA Cell Biol.* 14:945-50 (1995)); LacI mutants that respond to the presence of alternative sugars other than lactose in order to achieve induction, e.g., arabinose, ribose, or galactose (see, e.g., WO 99/27108 for Lac Repressor Proteins with Altered Responsivity); and LacI mutants that exhibit at least wild-type binding to lac operators, but enhanced sensitivity to an inducer (e.g., IPTG), or that exhibit enhanced binding to lac operators, but at least wild-type de-repressibility (see, e.g., L Swint-Kruse et al., *Biochemistry* 42(47):14004-16 (2003)).

[0176] In a particular embodiment, the gene encoding the Lac repressor protein inserted into the chromosome is identical to that of native *E.coli* lacI gene, and has the nucleic acid sequence of SEQ ID NO. 9 (Table 10). In another embodiment, the gene inserted into the host chromosome encodes the Lac repressor protein having the amino acid sequence of SEQ ID NO. 10 (Table 1 1).

TABLE 10

NUCLEIC ACID SEQUENCE OF NATIVE *E. COLI* LACI GENE

Gacaccatcgaatggcgcaaaacctttcgcggtatggcatgatagcgcccggaagagagtca	SEQ ID NO 9
attcagggtggtgaatgtgaaaccagtaacgttatacgatgtcgcagagtatgccggtgtct	
cttatcagaccgtttcccgcgtggtgaaccaggccagccacgtttctgcgaaaacgcgggaa	
aaagtggaagcggcgatggcggagctgaattacattcccaaccgcgtggcacaacaactggc	
gggcaaacagtcgttgctgattggcgttgccacctccagtctggccctgcacgcgccgtcgc	
aaattgtcgcggcgattaaatctcgcgccgatcaactgggtgccagcgtggtggtgtcgatg	
gtagaacgaagcggcgtcgaagcctgtaaagcggcggtgcacaatcttctcgcgcaacgcgt	
cagtgggctgatcattaactatccgctggatgaccaggatgccattgctgtggaagctgcct	
gcactaatgttccggcgttatttcttgatgtctctgaccagacacccatcaacagtattatt	
ttctcccatgaagacggtacgcgactgggcgtggagcatctggtcgcattgggtcaccagca	
aatcgcgctgttagcgggcccattaagttctgtctcggcgcgtctgcgtctggctggctggc	
ataaatatctcactcgcaatcaaattcagccgatagcggaacgggaaggcgactggagtgcc	
atgtccggttttcaacaaaccatgcaaatgctgaatgagggcatcgttcccactgcgatgct	
ggttgccaacgatcagatggcgctgggcgcaatgcgcgccattaccgagtccgggctgcgcg	
ttggtgcggatatctcggtagtgggatacgacgataccgaagacagctcatgttatatcccg	
ccgtcaaccaccatcaaacaggattttcgcctgctggggcaaaccagcgtggaccgcttgct	
gcaactctctcagggccaggcggtgaagggcaatcagctgttgcccgtctcactggtgaaaa	

TABLE 10-continued

NUCLEIC ACID SEQUENCE OF NATIVE *E. COLI* LACI GENE
gaaaaaccaccctggcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcatta
atgcagctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatg
tgagttagctcactcattaggcaccccaggctttacactttatgcttccggctcgtatgttg
tgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacggatt
cactggccgtcgttttacaacgtcgtga

[0177]

TABLE 11

AMINO ACID SEQUENCE OF LACI REPRESSOR
Met Lys Pro Val Thr Leu Tyr Asp Val Ala Glu Tyr Ala Gly Val SEQ ID NO. 10
Ser Tyr Gln Thr Val Ser Arg Val Val Asn Gln Ala Ser His Val
Ser Ala Lys Thr Arg Glu Lys Val Glu Ala Ala Met Ala Glu Leu
Asn Tyr Ile Pro Asn Arg Val Ala Gln Gln Leu Ala Gly Lys Gln
Ser Leu Leu Ile Gly Val Ala Thr Ser Ser Leu Ala Leu His Ala
Pro Ser Gln Ile Val Ala Ala Ile Lys Ser Arg Ala Asp Gln Leu
Gly Ala Ser Val Val Val Ser Met Val Glu Arg Ser Gly Val Glu
Ala Cys Lys Ala Ala Val His Asn Leu Leu Ala Gln Arg Val Ser
Gly Leu Ile Ile Asn Tyr Pro Leu Asp Asp Gln Asp Ala Ile Ala
Val Glu Ala Ala Cys Thr Asn Val Pro Ala Leu Phe Leu Asp Val
Ser Asp Gln Thr Pro Ile Asn Ser Ile Phe Ser His Glu Asp Gly
Thr Arg Leu Gly Val Glu His Leu Val Ala Leu Gly His Gln Gln
Ile Ala Leu Leu Ala Gly Pro Leu Ser Ser Val Ser Ala Arg Leu
Arg Leu Ala Gly Trp His Lys Tyr Leu Thr Arg Asn Gln Ile Gln
Pro Ile Ala Glu Arg Glu Gly Asp Trp Ser Ala Met Ser Gly Phe
Gln Gln Thr Met Gln Met Leu Asn Glu Gly Ile Val Pro Thr Ala
Met Leu Val Ala Asn Asp Gln Met Ala Leu Gly Ala Met Arg Ala
Ile Thr Glu Ser Gly Leu Arg Val Gly Ala Asp Ile Ser Val Val
Gly Tyr Asp Asp Thr Glu Asp Ser Ser Cys Tyr Ile Pro Pro Ser
Thr Thr Ile Lys Gln Asp Phe Arg Leu Leu Gly Gln Thr Ser Val
Asp Arg Leu Leu Gln Leu Ser Gln Gly Gln Ala Val Lys Gly Asn
Gln Leu Leu Pro Val Ser Leu Val Lys Arg Lys Thr Thr Leu Ala
Pro Asn Thr Gln Thr Ala Ser Pro Arg Ala Leu Ala Asp Ser Leu
Met Gln Leu Ala Arg Gln Val Ser Arg Leu Glu Ser Gly Gln

[0178] In an alternative embodiment, the inserted lacI transgene is a derivative of the native *E.coli* lacI gene. In one particular embodiment, the lacI derivative gene is the $lacI^{Q}$ gene having the nucleic acid sequence of SEQ ID NO. 11 (Table 12). The $lacI^{Q}$ variant is identical to the native *E.coli* lacI gene except that it has a single point mutation in the −35 region of the promoter which increases the level of lacI repressor by 10-fold in *E.coli* . See, for example, M P Calos, Nature 274 (5673): 762-65 (1978).

TABLE 12

NUCLEIC ACID SEQUENCE OF LACI^{Q} GENE

gacaccatcgaatggtgcaaaacctttcgcggtatggcatgatagcgcccggaagagagtca SEQ ID NO. 11

attcagggtggtgaatgtgaaaccagtaacgttatacgatgtcgcagagtatgccggtgtct

cttatcagaccgtttcccgcgtggtgaaccaggccagccacgtttctgcgaaaacgcgggaa

aaagtggaagcggcgatggcggagctgaattacattcccaaccgcgtggcacaacaactggc

gggcaaacagtcgttgctgattggcgttgccacctccagtctggccctgcacgcgccgtcgc

aaattgtcgcggcgattaaatctcgcgccgatcaactgggtgccagcgtggtggtgtcgatg

gtagaacgaagcggcgtcgaagcctgtaaagcggcggtgcacaatcttctcgcgcaacgcgt

cagtgggctgatcattaactatccgctggatgaccaggatgccattgctgtggaagctgcct

gcactaatgttccggcgttatttcttgatgtctctgaccagacacccatcaacagtattatt

ttctcccatgaagacggtacgcgactgggcgtggagcatctggtcgcattgggtcaccagca

aatcgcgctgttagcgggcccattaagttctgtctcggcgcgtctgcgtctggctggctggc

ataaatatctcactcgcaatcaaattcagccgatagcggaacgggaaggcgactggagtgcc

atgtccggttttcaacaaaccatgcaaatgctgaatgagggcatcgttcccactgcgatgct

ggttgccaacgatcagatggcgctgggcgcaatgcgcgccattaccgagtccgggctgcgcg

ttggtgcggatatctcggtagtgggatacgacgataccgaagacagctcatgttatatcccg

ccgtcaaccaccatcaaacaggattttcgcctgctggggcaaaccagcgtggaccgcttgct

gcaactctctcagggccaggcggtgaagggcaatcagctgttgcccgtctcactggtgaaaa

gaaaaaccaccctggcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcatta

atgcagctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatg

tgagttagctcactcattaggcaccccaggctttacactttatgcttccggctcgtatgttg

tgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacggatt

cactggccgtcgttttac

[0179] In still another embodiment, the lacI derivate gene is the lacI^{Q1} gene having the nucleic acid sequence of SEQ ID NO. 12 (Table 13). The lacI^{Q1} variant has a rearrangement which substitutes a −35 region whose nucleotide sequence exactly matches that of the *E.coli*−35 region consensus sequence, resulting in expression that is 100-fold higher than the native promoter in *E.coli*. See, for example, M P Colas & J H Miller, Mol. & Gen. Genet. 183(3): 559-60(1980).

TABLE 13

NUCLEIC ACID SEQUENCE OF LACI^{Q1} GENE

agcggcatgcatttacgttgacaccacctttcgcggtatggcatgatagcgcccggaagaga SEQ ID NO. 12

gtcaattcagggtggtgaatgtgaaaccagtaacgttatacgatgtcgcagagtatgccggt

gtctcttatcagaccgtttcccgcgtggtgaaccaggccagccacgtttctgcgaaaacgcg

ggaaaaagtggaagcggcgatggcggagctgaattacattcccaaccgcgtggcacaacaac

tggcgggcaaacagtcgttgctgattggcgttgccacctccagtctggccctgcacgcgccg

tcgcaaattgtcgcggcgattaaatctcgcgccgatcaactgggtgccagcgtggtggtgtc

gatggtagaacgaagcggcgtcgaagcctgtaaagcggcggtgcacaatcttctcgcgcaac

gcgtcagtgggctgatcattaactatccgctggatgaccaggatgccattgctgtggaagct

TABLE 13-continued

NUCLEIC ACID SEQUENCE OF LACI^{Q1} GENE

gcctgcactaatgttccggcgttatttcttgatgtctctgaccagacacccatcaacagtat

tattttctcccatgaagacggtacgcgactgggcgtggagcatctggtcgcattgggtcacc

agcaaatcgcgctgttagcgggcccattaagttctgtctcggcgcgtctgcgtctggctggc

tggcataaatatctcactcgcaatcaaattcagccgatagcggaacgggaaggcgactggag

tgccatgtccggttttcaacaaaccatgcaaatgctgaatgagggcatcgttcccactgcga

tgctggttgccaacgatcagatggcgctgggcgcaatgcgcgccattaccgagtccgggctg

cgcgttggtgcggatatctcggtagtgggatacgacgataccgaagacagctcatgttatat

cccgccgtcaaccaccatcaaacaggattttcgcctgctggggcaaaccagcgtggaccgct

tgctgcaactctctcagggccaggcggtgaagggcaatcagctgttgcccgtctcactggtg

aaaagaaaaaccaccctggcgcccaatacgcaaaccgcctctccccgcgcgttggccgattc

attaatgcagctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaatt

aatgtgagttagctcactcattaggcaccccaggctttacactttatgcttccggctcgtat

gttgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacg

gattcactggccgtcgttttac

[0180] In the present invention, the host cell chromosome can be modified by insertion of at least one nucleic acid sequence containing at least one copy of a gene encoding a LacI protein, the gene being capable of use by the cell to, preferably, constitutively express the encoded LacI protein, and the polynucleotide containing the gene being other than a PlacI-lacI-lacZYA nucleic acid sequence (i.e. a Plac(−) version of the PlacI-lacI-lacZYA operon) or a PlacI-lacI-lacZ polynucleotide (i.e. a structural lac utilization operon gene-containing portion of such a Plac(−) operon, such as an at least partially truncated version of a PlacI-lacI-lacZYA nucleic acid sequence).

[0181] The gene encoding the chosen LacI protein is preferably constitutively expressed. This may be accomplished by use of any promoter that is constitutively expressed in the selected expression host cell. For example, a native *E.coli* PlacI may be operably attached to the selected LacI coding sequence, or a different constitutively expressed promoter may be operably attached thereto. In some cases, a regulated promoter may be used, provided that the regulated promoter is maintained throughout fermentation in a state wherein the LacI protein is continually expressed there from. In a particular embodiment, a lac or tac family promoter is utilized in the present invention, including Plac, Ptac, Ptrc, PtacII, PlacUV5, 1 pp-PlacUV5, lpp-lac, nprM-lac, T71ac, T5lac, T3lac, and Pmac.

[0182] Genomic Insertion Sites

[0183] Chromosomal insertion may be performed according to any technique known in the art. For example, see: D S Toder, "Gene replacement in *Pseudomonas aeruginosa*," *Methods in Enzymology* 235:466-74 (1994); and J Quandt & M F Hynes, "Versatile suicide vectors which allow direct selection for gene replacement in Gram negative bacteria," *Gene* 127(1):15-21 (1993). Transposon-type insertion techniques such as are known in the art, followed by selection, may also be used; see, e.g., I Y Goryshin & W S Reznikoff, "Tn5 in vitro transposition," *Journal of Biological Chemistry* 273(13):7367-74 (1998). Alternatively, gene transfection by (non-lytic) phage transduction may also be used for chromosomal insertion; see, e.g., J H Miller, *Experiments in Molecular Genetics* (1972) (Cold Spring Harbor Lab., N.Y.).

[0184] Sites within the bacterial expression host cell chromosome that are useful places in which to insert the lacI gene(s), or derivative thereof, include any location that is are not required for cell function under the fermentation conditions used, for example within any gene whose presence, transcription, or expression is important for the healthy functioning of the cell under the fermentation conditions used. Illustrative examples of such insertion sites include, but are not limited to: sucrose import and metabolism genes (e.g., sacB), fructose import and catabolism genes (e.g., fructokinase genes, 1-phosphofructokinase genes), aromatic carbon source import and utilization genes (e.g., anthranilate operon genes, such as antABC genes, benzoate operon genes, as benABCD genes), beta-lactamase genes (e.g., ampC, bll1, blc genes, blo genes, blp genes), alkaline phosphatase genes (e.g., phoA), nucleobase or nucleotide biosynthetic genes (e.g., pyrBCDEF genes), amino acid biosynthetic genes (e.g., proABC genes), aspartate semi-aldehyde dehydrogenase genes (e.g., asd), 3-isopropylmalate dehydrogenase genes (e.g., leuB), and anthranilate synthase genes (e.g., trpE).

[0185] In any embodiment in which the genomic insertion has resulted in or is concomitant with an auxotrophy, then either the host cell will be grown in media supplying an effective replacement metabolite to the cell to overcome (and avoid) the lethal effect, or a replacement gene will be provided in the host cell that expresses a biocatalyst effective to restore the corresponding prototrophy, e.g., as a selection

marker gene. The gene or genes selected for deletion or inactivation (i.e. "knock-out") in constructing a metabolic auxotroph can be any gene encoding an enzyme that is operative in a metabolic pathway. The enzyme can be one that is involved in the anabolic biosynthesis of molecules that are necessary for cell survival. Alternatively, the enzyme can be one that is involved in the catabolic utilization of molecules that are necessary for cell survival. Preferably, all operative genes encoding a given biocatalytic activity are deleted or inactivated in order to ensure removal of the targeted enzymatic activity from the host cell in constructing the auxotrophic host cell. Alternatively, the host cell can exhibit a pre-existing auxotrophy (i.e. native auxotrophy), wherein no further genetic modification via deletion or inactivation (knock-out) need be performed.

[**0186**] For example, an amino acid biosynthetic gene (e.g., a proA, proB, or proC gene) or a nucleobase or nucleotide biosynthetic gene (e.g., pyrB, pyrC, pyrD, pyrE, or pyrF) may be used as the insertion site, in which case a necessary biosynthetic activity is normally disrupted, thus producing an auxotrophy. In such a case, either: 1) the medium is supplemented to avoid metabolic reliance on the biosynthetic pathway, as with a proline or uracil supplement; or 2) the auxotrophic host cell is transformed with a further gene that is expressed and thus replaces the biocatalyst(s) missing from the biosynthetic pathway, thereby restoring prototrophy to the cell, as with a metabolic selection marker gene such as proC, pyrF, or thyA. In a particular embodiment, the lacI transgene, or variant thereof, is inserted into a cell that is concomitantly or subsequently auxotrophically induced through the knock-out of a gene, or combination of genes, selected from the group consisting of pyrF, thyA, and proC. In a specific embodiment, a native *E.coli* lacI, $lacI^{Q}$, or $lacI^{Q1}$ transgene is inserted into a cell that is concomitantly or subsequently rendered auxotrophic through the knock-out of pyrF. In another specific embodiment, a native *E.coli* lacI, $lacI^{Q}$,or $lacI^{Q1}$ transgene is inserted into a cell that is concomitantly of subsequently rendered auxotrophic through the knock-out of proC. In still a further embodiment, a native *E.coli* lacI, $lacI^{Q}$, or $lacI^{Q1}$ transgene is inserted into a cell that is concomitantly or subsequently rendered auxotrophic through the knock-out of pyrF and proC.

[**0187**] In another embodiment, a native *E.coli* lacI, $lacI^{Q}$, or $lacI^{Q1}$ transgene, or derivative thereof, can be inserted into the Levansucrase locus of the host cell. For example, in one particular embodiment, a native *E.coli* lacI, $lacI^{Q}$, or $lacI^{Q1}$ transgene, or derivative thereof, can be inserted in the Levansucrase gene locus of *Pseudomonas fluorescens.* In particular, a native *E.coli* lacI, $lacI^{Q}$, or $lacI^{Q1}$ transgene, or derivative thereof, can be inserted into the Levansucrase gene locus of *Pseudomonas fluorescens* having the nucleic acid sequence of SEQ ID. NO. 13 (Table 14).

TABLE 14

OPEN READING FRAME OF PF LEVAN SUCRASE GENE LOCUS

ctacccagaacgaagatcagcgcctcaatggcctcaaggttctactggtcgatgattcagcc SEQ ID NO. 13

gaagtcgttgaggtgctgaacatgctgctggaaatggaaggcgcccaagtgagcgccttcag

cgacccttgagcgcgcttgaaacagcccgggatgcccattacgacgtgattatttcggaca

tcggcatgccgaaaatgaatggccatgagctgatgcagaagctgcgtaaagtaggccacctt

cgacaggctcccgccatcgccttaacgggctatggcgctggcaatgaccagaaaaaggcgac

tgaatcgggctttaatgcgcatgtcagcaaacccgttggccatgattcgctcatcaccttga

tcgaaaaactgtgccgctcccgcccctaggcgtggggcaggcgttcaagggtagatgaactg

agaaaagcgcacggacgcgcccgtttctggtcgcgacacctgggtatccacgctgcccaccg

tgtcgctgcgcaaggtcaggtacaacacggcctggccggcgctgtcactcagcatccagacg

ctcacaccctccccggccgccctggccttgagcggctgaggctgcagcatctcgatattgaa

accgcgcagcagctcaccgctcaactcgacctccaggggttcctgggccttaccttgcacat

gaatcaccagcccatcggaggcgccattgcgcaaaaagcgttggtactccacgcgcaactgc

ccatcggcactgcgcacctcgcggctgctcagcggcccgctggaaaacagccctgccaagct

caagccgatcagcaccagcagcgcgtaccaacccacccgctcaaagcgccagaccttgcgct

gcaaggccatgttttcctgcaccggataattgcggctgtgtaagtcgtcagggtctgggttg

ttcatagcggggcccggactcaacccttgctgtgctcgggagaagacggccccttggtgaca

ccccgtgggccggcaatcgcccatatcgcagcgcccagaaacggcagcaccacgactaccgc

actccagcctgccttgctggccgaggcgttatcgctgcgccagatgctgttgatgatccacg

catcgagcagtacgaggatcactgccaggcctatccagaagtaagtggtttgcatgatgcac

ctccaggttatgtaacttttggtgcgcgggtgcgggcagggttcattatttttaggttctct

TABLE 14-continued

OPEN READING FRAME OF PF LEVAN SUCRASE GENE LOCUS

gcctggcgcttggtttgccgccatcatgcgggcaacttcgccgatctacttaatgatcgaac

ctcttcaaacaagacaagctgaaacgtctcagctcctataaaaagccaaatcatgcacaaat

gcatttttgccttgaccacgggaatcgagtcttctaaagtcaaatcactgtatatgaatac

agtaatttgattcccttcatggacgagacttactatgaaaagcacccttcgaaatttggca

aaacaccccatcaacccagcctgtggacccgcgccgatgcgcttaaagtgcatgcggacgac

cccaccaccacccagccgctggtcagcgcgaacttcccggtattgagtgacgaggtgtttat

ctgggacaccatgccgctgcgtgatatcgacggcaacatcacctccgtcgatggctggtcgg

tgatcttcaccctcaccgcggatcgccacccgaacgacccgcaatacctcgatcagaatggc

aactacgacgtcatccgcgactggaacgatcgccatggccgggcaaagatgtactactggtt

ctcccgcaccggcaaagactggaagctcggcggccgagtgatggctgaaggggtttcgccca

ccgtgcgcgaatgggccggcacgccgatcctgttgaacgagcaaggcgaagtagacctgtac

tacaccgccgtcacgcccggcgcgaccatcgtcaaggtgcgtggccgcgtggtgaccaccga

gcatggcgtcagcctggtgggctttgagaaggtcaagccgctgttcgaggcggacggcaaga

tgtaccagaccgaagcgcaaaatgcgttctggggctttcgcgatccatggccgttccgcgac

ccgaaagacggcaagctgtacatgctgttcgaaggtaacgtggccggcgaacgcggctcgca

caaggtcggtaaagccgaaatcggcgacgtgccgccaggttatgaagacgtcggtaactcgc

gcttccagactgcctgcgtcggtatcgccgtggcccgcgacgaagacggcgacgactgggaa

atgctgccaccgctgctgaccgcggtgggcgtcaacgaccagaccgaacgcccgcacttcgt

gttccaggacggcaagtactacctgttcaccatcagccacaccttcacctacgccgacggcg

tgaccggcccggacggcgtgtacggcttcgtcgccgattcgctgttcggtccgtatgtgccg

ttgaacggctctggtctggtactgggcaacccgtcctcccaaccgttccagacctactcgca

ctgcgtcatgcccaacggcctggtgacctccttcatcgacagcgtaccgaccgacgacaccg

gcacgcagatccgtatcggcggcaccgaagcaccgacggtgggcatcaagatcaaagggcag

caaacgtttgtggtcgctgagtatgactacggttacatcccgccgatgctcgacgttacgct

caagtaaccggaggctatgaggtagcggctttgagctcgatgacaaacccgcggtgaatatt

cgctgcacctgtggcgagggagcttgctcccggttgggccggacagccgccatcgcaggcaa

gccagctcccacattttggttcctggggcgtcagggaggtatgtgtcggctgaggggccgtc

acgggagcaagctccctcgccacaggttcaacagcccattgggtggatattcaggaaataga

aatgcctgcaccattgagttgagtc

IV. LacO Sequences

[0188] Attempts to repress the leakiness of a promoter must be balanced by the potential concomitant reduction in target recombinant polypeptide expression. One approach to further repress a promoter and reduce the leakiness of the promoters is to modify regulatory elements known as operator sequences, to increase the capacity of the associated repressor protein to bind to the operator sequence without reducing the potential expression of the target recombinant polypeptide upon induction.

[0189] It has been discovered that the use of a dual lac operator in *Pseudomonas fluorescens* offers superior repression of pre-induction recombinant protein expression without concomitant reductions in induced protein yields.

[0190] In one embodiment, a Pseudomonad organism is provided comprising a nucleic acid construct containing a nucleic acid comprising at least one lacO sequence involved in the repression of transgene expression. In a particular embodiment, the Pseudomonad host cell is *Pseudomonad fluorescens*. In one embodiment, the nucleic acid construct comprises more than one lacO sequence. In another embodi-

ment, the nucleic acid construct comprises at least one, and preferably more than one, lacOid sequence. In one embodiment, the nucleic acid construct comprises a lacO sequence, or derivative thereof, located 3' of a promoter, and a lacO sequence, or derivative thereof, located 5' of a promoter. In a particular embodiment, the lacO derivative is a lacOid sequence.

[0191] In another embodiment of the present invention, nucleic acid constructs comprising more than one lac operator sequence, or derivative thereof for use in a Pseudomonad host cell is provided. In one embodiment, at least one lac operator sequence may be a $lacO_{id}$ sequence.

[0192] The native *E.coli* lac operator acts to down regulate expression of the lac operon in the absence of an inducer. To this end, the lac operator is bound by the LacI repressor protein, inhibiting transcription of the operon. It has been determined that the LacI protein can bind simultaneously to two lac operators on the same DNA molecule. See, for example, Muller et al., (1996) "Repression of lac promoter as a function of distance, phase, and quality of an auxiliary lac operator," J.Mol.Biol. 257: 21-29. The repression is mediated by the promoter-proximal operator O_1 and the two auxiliary operators O_2 and O_3, located 401 base pairs downstream of O_1 within the coding region of the lacZ gene and 92 bp upstream of O_1, respectively (See **FIG. 4**). Replacement of the native *E.coli* lac operator sequences with an ideal lac operator (O_{id}) results in increased repression of the native lac operon in *E.coli* . See Muller et al., (1996) "Repression of lac promoter as a function of distance, phase, and quality of an auxiliary lac operator," J.Mol.Biol. 257: 21-29.

[0193] The lacO sequence or derivative can be positioned in the *E.coli* native O_1 position with respect to a promoter. Alternatively, the lacO sequence or derivative can be positioned in the *E.coli* O_3 position with respect to a promoter. In another embodiment, the lacO sequence or derivative can be located in the *E.coli* native O_1 position, the native O_3 position, or both with respect to a promoter. In one embodiment, the nucleic acid construct contains at least one lacOid sequence either 5' to the promoter sequence or 3' to the promoter sequence. In a particular embodiment, the nucleic acid construct contains a lacOid sequence 3' of a promoter, and at least one lacO sequence, or derivative, 5' of a promoter. In an alternative embodiment, the nucleic acid construct contains a lacOid sequence 5' of a promoter, and at least one lacO sequence, or derivative, 3' of a promoter. In still another embodiment, the nucleic acid construct contains a lacOid sequence both 5' and 3' of a promoter.

[0194] In a particular embodiment, the laco sequence is lacOid represented by SEQ ID NO. 14, or a sequence substantially homologous. In another embodiment, a lacOid sequence of SEQ. ID. NO. 59, or a sequence substantially homologous to SEQ ID NO. 59 is employed.

TABLE 15

LACOID SEQUENCE	
5'-AATTGTGAGCGCTCACAATT-3'	SEQ ID NO. 14
5'-tgtgtggAATTGTGAGCGCTCACAATTccacaca-3'	SEQ ID NO. 59

[0195] V. Isolated Nucleic Acids and Amino Acids

[0196] In another aspect of the present invention, nucleic acid sequences are provided for use in the improved production of proteins.

[0197] In one embodiment, nucleic acid sequences encoding prototrophy-restoring enzymes for use in an auxotrophic Pseudomonad host cells are provided. In a particular embodiment, nucleic acid sequences encoding nitrogenous base compound biosynthesis enzymes purified from the organism *Pseudomonas fluorescens* are provided. In one embodiment, nucleic acid sequences encoding the pyrF gene in *Pseudomonas fluorescens* is provided (SEQ. ID No.s 1 and 3). In another embodiment, a nucleic acid sequence encoding the thyA gene in *Pseudomonas fluorescens* is provided (SEQ. ID. No. 4). In still another embodiment, nucleic acid sequences encoding an amino acid biosynthetic compound purified from the organism *Pseudomonas fluorescens* are provided. In a particular embodiment, a nucleic acid sequence encoding the proC gene in *Pseudomonas fluorescens* is provided (SEQ. ID No.s 6 and 8).

[0198] In another aspect, the present invention provides novel amino acid sequences for use in the improved production of proteins.

[0199] In one embodiment, amino acid sequences of nitrogenous base compound biosynthesis enzymes purified from the organism *Pseudomonas fluorescens* are provided. In one embodiment, the amino acid sequence containing SEQ. ID No. 2 is provided. In another embodiment, an amino acid sequence containing SEQ. ID. No. 5 is provided. In still another embodiment, amino acid sequences of an amino acid biosynthetic compound purified from the organism *Pseudomonas fluorescens* is provided. In a particular embodiment, an amino acid sequence containing SEQ. ID No. 7 is provided.

[0200] One embodiment of the present invention is novel isolated nucleic acid sequences of the *Pseudomonas fluorescens* pyrF gene (Table 2, Seq. ID No. 1; Table 4, Seq. ID No. 3). Another aspect of the present invention provides isolated peptide sequences of the *Pseudomonas fluorescens* pyrF gene (Table 3, Seq. ID No. 2). Nucleic and amino acid sequences containing at least 90, 95, 98 or 99% homologous to Seq. ID Nos. 1, 2, or 3 are provided. In addition, nucleotide and peptide sequences that contain at least 10, 15, 17, 20 or 25, 30, 40, 50, 75, 100, 150, 250, 350, 500, or 1000 contiguous nucleic or amino acids of Seq ID Nos 1, 2, or 3 are also provided. Further provided are fragments, derivatives and analogs of Seq. ID Nos. 1, 2, or 3. Fragments of Seq. ID Nos. 1, 2, or 3 can include any contiguous nucleic acid or peptide sequence that includes at least about 10 bp, 15 bp, 17 bp, 20 bp, 50 bp, 100 bp, 500 bp, 1 kbp, 5 kbp or 10 kpb.

[0201] Another embodiment of the present invention is novel isolated nucleic acid sequences of the *Pseudomonas fluorescens* thyA gene (Table 5, Seq. ID No. 4). Another aspect of the present invention provides isolated peptide sequences of the *Pseudomonas fluorescens* thyA gene (Table 6, Seq. ID No. 5). Nucleic and amino acid sequences containing at least 90, 95, 98 or 99% homologous to Seq. ID Nos. 4 or 5 are provided. In addition, nucleotide and peptide sequences that contain at least 10, 15, 17, 20 or 25, 30, 40, 50, 75, 100, 150, 250, 350, 500, or 1000 contiguous nucleic

or amino acids of Seq ID Nos 4 or 5 are also provided. Further provided are fragments, derivatives and analogs of Seq. ID Nos. 4 or 5. Fragments of Seq. ID Nos. 4 or 5 can include any contiguous nucleic acid or peptide sequence that includes at least about 10 bp, 15 bp, 17 bp, 20 bp, 50 bp, 100 bp, 500 bp, 1 kbp, 5 kbp or 10

[0202] Another embodiment of the present invention is novel isolated nucleic acid sequences of the *Pseudomonas fluorescens* proC gene (Table 7, Seq. ID No. 6; Table 9, Seq. ID. No. 8). Another aspect of the present invention provides isolated peptide sequences of the *Pseudomonas fluorescens* proC gene (Table 8, Seq. ID No. 7). Nucleic and amino acid sequences containing at least 90, 95, 98 or 99% homologous to Seq. ID Nos. 6, 7, or 8 are provided. In addition, nucleotide and peptide sequences that contain at least 10, 15, 17, 20 or 25, 30, 40, 50, 75, 100, 150, 250, 350, 500, or 1000 contiguous nucleic or amino acids of Seq ID Nos 6, 7, or 8 are also provided. Further provided are fragments, derivatives and analogs of Seq. ID Nos. 6, 7, or 8. Fragments of Seq. ID Nos. 6, 7, or 8 can include any contiguous nucleic acid or peptide sequence that includes at least about 10 bp, 15 bp, 17 bp, 20 bp, 50 bp, 100 bp, 500 bp, 1 kbp, 5 kbp or 10 kpb.

[0203] Sequence Homology

[0204] Sequence homology is determined according to any of various methods well known in the art. Examples of useful sequence alignment and homology determination methodologies include those described below.

[0205] Alignments and searches for homologous sequences can be performed using the U.S. National Center for Biotechnology Information (NCBI) program, Mega-BLAST (currently available at http://www.ncbi.nlm.nih-.gov/BLAST/). Use of this program with options for percent identity set at 70% for amino acid sequences, or set at 90% for nucleotide sequences, will identify those sequences with 70%, or 90%, or greater homology to the query sequence. Other software known in the art is also available for aligning and/or searching for homologous sequences, e.g., sequences at least 70% or 90% homologous to an information string containing a promoter base sequence or activator-protein-encoding base sequence according to the present invention. For example, sequence alignments for comparison to identify sequences at least 70% or 90% homologous to a query sequence can be performed by use of, e.g., the GAP, BESTFIT, BLAST, FASTA, and TFASTA programs available in the GCG Sequence Analysis Software Package (available from the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705), with the default parameters as specified therein, plus a parameter for the extent of homology set at 70% or 90%. Also, for example, the CLUSTAL program (available in the PC/Gene software package from Intelligenetics, Mountain View, Calif.) may be used.

[0206] These and other sequence alignment methods are well known in the art and may be conducted by manual alignment, by visual inspection, or by manual or automatic application of a sequence alignment algorithm, such as any of those embodied by the above-described programs. Various useful algorithms include, e.g.: the similarity search method described in W. R. Pearson & D. J. Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444-48 (Apr 1988); the local homology method described in T. F. Smith & M. S. Waterman, in *Adv. Appl. Math.* 2:482-89 (1981) and in *J. Molec. Biol.* 147:195-97 (1981); the homology alignment method described in S. B. Needleman & C. D. Wunsch, *J. Molec. Biol.* 48(3):443-53 (Mar 1970); and the various methods described, e.g., by W. R. Pearson, in *Genomics* 11(3):635-50 (Nov 1991); by W. R. Pearson, in *Methods Molec. Biol.* 24:307-31 and 25:365-89 (1994); and by D. G. Higgins & P. M. Sharp, in *Comp. Appl'ns in Biosci.* 5:151-53 (1989) and in *Gene* 73(1):237-44 (15 Dec 1988).

[0207] Nucleic acid hybridization performed under highly stringent hybridization conditions is also a useful technique for obtaining sufficiently homologous sequences for use herein.

VI. Nucleic Acid Constructs

[0208] In still another aspect of the present invention, nucleic acid constructs are provided for use in the improved production of peptides.

[0209] In one embodiment, a nucleic acid construct for use in transforming a Pseudomonad host cell comprising a) a nucleic acid sequence encoding a recombinant polypeptide, and b) a nucleic acid sequence encoding a prototrophy-enabling enzyme is provided. In another embodiment, the nucleic acid construct further comprises c) a Plac-Ptac family promoter. In still another embodiment, the nucleic acid construct further comprises d) at least one lacO sequence, or derivative, 3' of a lac or tac family promoter. In yet another embodiment, the nucleic acid construct further comprises e) at least one lacO sequence, or derivative, 5' of a lac or tac family promoter. In one embodiment, the derivative lacO sequence can be a lacOid sequence. In a particular embodiment, the Pseudomonad organism is *Pseudomonas fluorescens.*

[0210] In one embodiment of the present invention, nucleic acid constructs are provided for use as expression vectors in Pseudomonad organisms comprising a) a nucleic acid sequence encoding a recombinant polypeptide, b) a Plac-Ptac family promoter, c) at least one lacO sequence, or derivative, 3' of a lac or tac family promoter, d) at least one lacO sequence, or derivative, 5' of a lac or tac family promoter. In one embodiment, the derivative lacO sequence can be a lacOid sequence. In one embodiment, the nucleic acid construct further comprises e) a prototrophy-enabling selection marker for use in an auxotrophic Pseudomonad cell. In a particular embodiment, the Pseudomonad organism is *Pseudomonas fluorescens.*

[0211] In one embodiment of the present invention, a nucleic acid construct is provided comprising nucleic acids that encode at least one biosynthetic enzyme capable of transforming an auxotrophic host cell to prototrophy. The biosynthetic enzyme can be any enzyme capable of allowing an auxotrophic host cell to survive on a selection medium that, without the expression of the biosynthetic enzyme, the host cell would be incapable of survival due to the auxotrophic metabolic deficiency. As such, the biosynthetic enzyme can be an enzyme that complements the metabolic deficiency of the auxotrophic host by restoring prototrophic ability to grow on non-auxotrophic metabolite supplemented media.

[0212] In one particular embodiment, the present invention provides a nucleic acid construct that encodes a func-

tional orotodine-5'-phosphate decarboxylase enzyme that complements an pyrF(−) auxotrophic host. In a particular embodiment, the nucleic acid construct contains the nucleic acid sequence of SEQ ID NO. 1 or 3. In an alternative embodiment, the nucleic acid construct contains a nucleic acid sequence that encodes the amino acid sequence of SEQ ID NO. 2.

[0213] In another particular embodiment, the present invention provides a nucleic acid construct that encodes a functional thymidylate synthase enzyme that complements a thyA (−) auxotrophic host. In a particular embodiment, the nucleic acid construct contains the nucleic acid sequence of SEQ ID NO. 4. In an alternative embodiment, the nucleic acid construct contains a nucleic acid sequence that encodes the amino acid sequence of SEQ ID NO. 5.

[0214] In a further particular embodiment, the present invention provides a nucleic acid construct that encodes a functional Δ^1-pyrroline-5-carboxylate reductase enzyme that complements a proC (−) auxotrophic host. In a particular embodiment, the nucleic acid construct contains the nucleic acid sequence of SEQ ID NO. 6 or 8. In an alternative embodiment, the nucleic acid construct contains the nucleic acid sequence that encodes the amino acid sequence of SEQ ID NO. 7.

[0215] In an alternative embodiment, the present invention provides a nucleic acid construct that encodes at least one biosynthetic enzyme capable of transforming an auxotrophic host cell to prototrophy and an additional non-auxotrophic selection marker. Examples of non-auxotrophic selection markers are well known in the art, and can include markers that give rise to colorimetric/chromogenic or a luminescent reaction such as lacZ gene, the GUS gene, the CAT gene, the luxAB gene, antibiotic resistance selection markers such as amphotericin B, bacitracin, carbapenem, cephalosporin, ethambutol, fluoroquinolones, isonizid, cephalosporin, methicillin, oxacillin, vanomycin, streptomycin, quinolines, rifampin, rifampicin, sulfonamides, ampicillin, tetracycline, neomycin, cephalothin, erythromycin, streptomycin, kanamycin, gentamycin, penicillin, and chloramphenicol resistance genes, or other commonly used non-auxotrophic selection markers.

[0216] In another embodiment, the expression vector can comprise more than one biosynthetic enzyme capable of transforming an auxotrophic host cell to prototrophy. The biosynthetic enzymes can be any enzymes capable of allowing an auxotrophic host cell to survive on a selection medium that, without the expression of the biosynthetic enzyme, the host cell would be incapable of survival due to the auxotrophic metabolic deficiency. As such, the biosynthetic enzymes can be enzymes that complement the metabolic deficiencies of the auxotrophic host by restoring prototrophic ability to grow on non-auxotrophic metabolite supplemented media. For example, an expression vector comprise a first and second prototrophy-enabling selection marker gene, allowing the host cell containing the construct to be maintained under either or both of the conditions in which host cell survival requires the presence of the selection marker gene(s). When only one of the marker-gene dependent survival conditions is present, the corresponding marker gene must be expressed, and the other marker gene(s) may then be either active or inactive, though all necessary nutrients for which the cell remains auxotrophic will still be supplied by the medium. This permits the same target gene, or the same set of covalently linked target genes, encoding the desired transgenic product(s) and/or desired transgenic activity(ies), to be maintained in the host cell continuously as the host cell is transitioned between or among different conditions. The coding sequence of each of the chosen selection marker genes independently can be operatively attached to either a constitutive or a regulated promoter.

[0217] In a particular embodiment, the nucleic acid vector comprises a nucleic acid construct that encodes a functional orotodine-5'-phosphate decarboxylase enzyme and a functional Δ^1-pyrroline-5-carboxylate reductase enzyme that can complement a pyrF(−) auxotrophic host cell, a proC(−) auxotrophic host cell, or a pyrF(−)/proC(−) dual-auxotrophic host cell. In a particular embodiment, the nucleic acid construct comprises the nucleic acid sequences of SEQ ID NO. 1 or 3, and SEQ ID. NO. 6 or 8. In an alternative embodiment, the nucleic acid construct contains a nucleic acid sequence that encodes the amino acid sequences of SEQ ID NO. 2 and 7.

[0218] In an alternative particular embodiment, the nucleic acid vector comprises a nucleic acid construct that encodes a functional orotodine-5'-phosphate decarboxylase enzyme and a functional thymidylate synthase enzyme that can complement a pyrF(−) auxotrophic host cell, a thyA(−) auxotrophic host cell, or a pyrF(−)/thyA(−) dual-auxotrophic host cell. In a particular embodiment, the nucleic acid construct comprises the nucleic acid sequences of SEQ ID NO. 1 or 3, and SEQ ID. NO. 4. In an alternative embodiment, the nucleic acid construct contains a nucleic acid sequence that encodes the amino acid sequences of SEQ ID NO. 2 and 5.

[0219] In a particular embodiment, the nucleic acid vector comprises a nucleic acid construct that encodes a functional Δ^1-pyrroline-5-carboxylate reductase enzyme and a thymidylate synthase enzyme that can complement a proC(−) auxotrophic host cell, a thyA(−) auxotrophic host cell, or a proC(−)/thyA(−) dual-auxotrophic host cell. In a particular embodiment, the nucleic acid construct comprises the nucleic acid sequences of SEQ ID NO. 4, and SEQ ID. NO. 6 or 8. In an alternative embodiment, the nucleic acid construct contains a nucleic acid sequence that encodes the amino acid sequences of SEQ ID NO. 5 and 7.

[0220] Promoters

[0221] In a fermentation process, once expression of the target recombinant polypeptide is induced, it is ideal to have a high level of production in order to maximize efficiency of the expression system. The promoter initiates transcription and is generally positioned 10-100 nucleotides upstream of the ribosome binding site. Ideally, a promoter will be strong enough to allow for recombinant polypeptide accumulation of around 50% of the total cellular protein of the host cell, subject to tight regulation, and easily (and inexpensively) induced.

[0222] The promoters used in accordance with the present invention may be constitutive promoters or regulated promoters. Examples of commonly used inducible promoters and their subsequent inducers include lac (IPTG), lacUV5 (IPTG), tac (IPTG), trc (IPTG), P_{syn} (IPTG), trp (tryptophan starvation), araBAD (1-arabinose), $1pp^a$ (IPTG), 1pp-lac

(IPTG), phoA (phosphate starvation), recA (nalidixic acid), proU (osmolarity), cst-1 (glucose starvation), teta (tretracylin), cada (pH), nar (anaerobic conditions), PL (thermal shift to 42° C.), cspA (thermal shift to 20° C.), T7 (thermal induction), T7-lac operator (IPTG), T3-lac operator (IPTG), T5-lac operator (IPTG), T4 gene32 (T4 infection), nprM-lac operator (IPTG), Pm (alkyl- or halo-benzoates), Pu (alkyl- or halo-toluenes), Psa1 (salicylates), and VHb (oxygen). See, for example, Makrides, S. C. (1996) Microbiol. Rev. 60, 512-538; Hannig G. & Makrides, S. C. (1998) TIBTECH 16, 54-60; Stevens, R. C. (2000) Structures 8, R177-R185. See, e.g.: J. Sanchez-Romero & V. De Lorenzo, Genetic Engineering of Nonpathogenic *Pseudomonas* strains as Biocatalysts for Industrial and Environmental Processes, in Manual of Industrial Microbiology and Biotechnology (A. Demain & J. Davies, eds.) pp.460-74 (1999) (ASM Press, Washington, D.C.); H. Schweizer, Vectors to express foreign genes and techniques to monitor gene expression for Pseudomonads, Current-Opinion in Biotechnology, 12:439-445 (2001); and R. Slater & R. Williams, The Expression of Foreign DNA in Bacteria, in Molecular Biology and Biotechnology (J. Walker & R. Rapley, eds.) pp.125-54 (2000) (The Royal Society of Chemistry, Cambridge, UK).

[**0223**] A promoter having the nucleotide sequence of a promoter native to the selected bacterial host cell can also be used to control expression of the transgene encoding the target polypeptide, e.g, a *Pseudomonas* anthranilate or benzoate operon promoter (Pant, Pben). Tandem promoters may also be used in which more than one promoter is covalently attached to another, whether the same or different in sequence, e.g., a Pant-Pben tandem promoter (interpromoter hybrid) or a Plac-Plac tandem promoter.

[**0224**] Regulated promoters utilize promoter regulatory proteins in order to control transcription of the gene of which the promoter is a part. Where a regulated promoter is used herein, a corresponding promoter regulatory protein will also be part of an expression system according to the present invention. Examples of promoter regulatory proteins include: activator proteins, e.g., *E.coli* catabolite activator protein, MalT protein; AraC family transcriptional activators ; repressor proteins, e.g., *E.coli* LacI proteins; and dual-faction regulatory proteins, e.g., *E.coli* NagC protein. Many regulated-promoter/promoter-regulatory-protein pairs are known in the art.

[**0225**] Promoter regulatory proteins interact with an effector compound, i.e. a compound that reversibly or irreversibly associates with the regulatory protein so as to enable the protein to either release or bind to at least one DNA transcription regulatory region of the gene that is under the control of the promoter, thereby permitting or blocking the action of a transcriptase enzyme in initiating transcription of the gene. Effector compounds are classified as either inducers or co-repressors, and these compounds include native effector compounds and gratuitous inducer compounds. Many regulated-promoter/promoter-regulatory-protein/effector-compound trios are known in the art. Although an effector compound can be used throughout the cell culture or fermentation, in a particular embodiment in which a regulated promoter is used, after growth of a desired quantity or density of host cell biomass, an appropriate effector compound is added to the culture in order to directly or indirectly result in expression of the desired target gene(s).

[**0226**] By way of example, where a lac family promoter is utilized, a lacI gene, or derivative thereof such as a $lacI^{Q}$ or $lacI^{Q1}$ gene, can also be present in the system. The lacI gene, which is (normally) a constitutively expressed gene, encodes the Lac repressor protein (LacI protein) which binds to the lac operator of these promoters. Thus, where a lac family promoter is utilized, the lacI gene can also be included and expressed in the expression system. In the case of the lac promoter family members, e.g., the tac promoter, the effector compound is an inducer, preferably a gratuitous inducer such as IPTG (isopropyl-β-D-1-thiogalactopyranoside, also called "isopropylthiogalactoside").

[**0227**] In a particular embodiment, a lac or tac family promoter is utilized in the present invention, including Plac, Ptac, Ptrc, PtacII, PlacUV5, Ipp-PlacUV5, Ipp-lac, nprM-lac, T71ac, T51ac, T31ac, and Pmac.

[**0228**] Other Elements

[**0229**] Other regulatory elements can be included in an expression construct, including lacO sequences and derivatives, as discussed above. Such elements include, but are not limited to, for example, transcriptional enhancer sequences, translational enhancer sequences, other promoters, activators, translational start and stop signals, transcription terminators, cistronic regulators, polycistronic regulators, tag sequences, such as nucleotide sequence "tags" and "tag" peptide coding sequences, which facilitates identification, separation, purification, or isolation of an expressed polypeptide, including His-tag, Flag-tag, T7-tag, S-tag, HSV-tag, B-tag, Strep-tag, polyarginine, polycysteine, polyphenylalanine, polyaspartic acid, (Ala-Trp-Trp-Pro)n, thioredoxin, beta-galactosidase, chloramphenicol acetyltransferase, cyclomaltodextrin gluconotransferase, CTP:CMP-3-deoxy-D-manno-octulosonate cytidyltransferase, trpE or trpLE, avidin, streptavidin, T7 gene 10, T4 gp55, Staphylococcal protein A, streptococcal protein G, GST, DHFR, CBP, MBP, galactose binding domain, Calmodulin binding domain, GFP, KSI, c-myc, ompT, ompA, pelB, , NusA, ubiquitin, and hemosylin A.

[**0230**] At a minimum, a protein-encoding gene according to the present invention can include, in addition to the protein coding sequence, the following regulatory elements operably linked thereto: a promoter, a ribosome binding site (RBS), a transcription terminator, translational start and stop signals. Useful RBSs can be obtained from any of the species useful as host cells in expression systems according to the present invention, preferably from the selected host cell. Many specific and a variety of consensus RBSs are known, e.g., those described in and referenced by D. Frishman et al., Starts of bacterial genes: estimating the reliability of computer predictions, Gene 234(2):257-65 (8 Jul. 1999); and B. E. Suzek et al., A probabilistic method for identifying start codons in bacterial genomes, Bioinformatics 17(12):1123-30 (December 2001). In addition, either native or synthetic RBSs may be used, e.g., those described in: EP 0207459 (synthetic RBSs); 0. Ikehata et al., Primary structure of nitrile hydratase deduced from the nucleotide sequence of a *Rhodococcus* species and its expression in *Escherichia* coli, Eur. J. Biochem. 181(3):563-70 (1989) (native RBS sequence of AAGGAAG). Further examples of

methods, vectors, and translation and transcription elements, and other elements useful in the present invention are described in, e.g.: U.S. Pat. No. 5,055,294 to Gilroy and U.S. Pat. No. 5,128,130 to Gilroy et al.; U.S. Pat. No. 5,281,532 to Rammler et al.; U.S. Pat. Nos. 4,695,455 and 4,861,595 to Barnes et al.; U.S. Pat. No.4,755,465 to Gray et al.; and U.S. Pat. No. 5,169,760 to Wilcox.

[0231] Vectors

[0232] Transcription of the DNA encoding the enzymes of the present invention by a Pseudomonad host can further be increased by inserting an enhancer sequence into the vector or plasmid. Typical enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp in size that act on the promoter to increase its transcription.

[0233] Generally, the recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the Pseudomonad host cell, e.g., the prototrophy restoring genes of the present invention, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters have been described above. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and in certain embodiments, a leader sequence capable of directing secretion of the translated polypeptide. Optionally, and in accordance with the present invention, the heterologous sequence can encode a fusion polypeptide including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

[0234] Useful expression vectors for use with *P. fluorescens* in expressing enzymes are constructed by inserting a structural DNA sequence encoding a desired target polypeptide together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable hosts for transformation in accordance with the present disclosure include various species within the genera *Pseudomonas,* and particularly particular is the host cell strain of *Pseudomonas fluorescens.*

[0235] Vectors are known in the art as useful for expressing recombinant proteins in host cells, and any of these may be modified and used for expressing the genes according to the present invention. Such vectors include, e.g., plasmids, cosmids, and phage expression vectors. Examples of useful plasmid vectors that can be modified for use on the present invention include, but are not limited to, the expression plasmids pBBR1MCS, pDSK519, pKT240, pML122, pPS10 , RK2, RK6, pRO1600, and RSF1010. Further examples can include pALTER-Ex1, pALTER-Ex2, pBAD/His, pBAD/Myc-His, pBAD/gIII, pCal-n, pCal-n-EK, pCal-c, pCal-Kc, pcDNA 2.1, pDUAL, pET-3a-c, pET 9a-d, pET-11a-d, pET-12a-c, pET-14b, pET15b, pET-16b, pET-17b, pET-19b, pET-20b(+), pET-21a-d(+), pET-22b(+), pET-23a-d(+), pET24a-d(+), pET-25b(+), pET-26b(+), pET-27b(+), pET28a-c(+), pET-29a-c(+), pET-30a-c(+), pET31b(+), pET-32a-c(+), pET-33b(+), pET-34b(+), pET35b(+), pET-36b(+), pET-37b(+), pET-38b(+), pET-39b(+), pET-40b(+), pET41la-c(+), pET-42a-c(+pET43a-c(+), pETBlue-1, pETBlue-2, pETBlue-3, pGEMEX-1, pGEMEX-2, pGEX1λT, pGEX-2T, pGEX-2TK, pGEX-3X, pGEX4T, pGEX-5X, pGEX-6P, pHAT10/11/12, pHAT20, pHAT-GFPuv, pKK223-3, pLEX, pMAL-c2X, pMAL-c2E, pMAL-c2g, pMAL-p2X, pMAL-p2E, pMAL-p2G, pProEX HT, pPROLar.A, pPROTet.E, pQE-9, pQE-16, pQE-30/31/32, pQE40, pQE-50, pQE-70, pQE-80/81/82L, pQE-100, pRSET, and pSE280, pSE380, pSE420, pThioHis, pTrc99A, pTrcHis, pTrcHis2, pTriEx-1, pTriEx-2, pTrxFus. Other examples of such useful vectors include those described by, e.g.: N. Hayase, in Appl. Envir. Microbiol. 60(9):3336-42 (September 1994); A. A. Lushnikov et al., in Basic Life Sci. 30:657-62 (1985); S. Graupner & W. Wackernagel, in Biomolec. Eng. 17(1):11-16. (October 2000); H. P. Schweizer, in Curr. Opin. Biotech. 12(5):439-45 (October 2001); M. Bagdasarian &. K. N. Timmis, in Curr. Topics Microbiol. Immunol. 96:47-67 (1982); T. Ishii et al., in FEMS Microbiol. Lett. 116(3):307-13 (Mar 1, 1994); I. N. Olekhnovich & Y. K. Fomichev, in Gene 140(1):63-65 (Mar 11, 1994); M. Tsuda & T. Nakazawa, in Gene 136(1-2):257-62 (Dec. 22, 1993); C. Nieto et al., in Gene 87(1):145-49 (Mar 1, 1990); J. D. Jones & N. Gutterson, in Gene 61(3):299-306 (1987); M. Bagdasarian et al., in Gene 16(1-3):237-47 (December 1981); H. P. Schweizer et al., in Genet. Eng. (NY) 23:69-81 (2001); P. Mukhopadhyay et al., in J. Bact. 172(1):477-80 (January 1990); D. O. Wood et al., in J. Bact. 145(3):1448-51 (March 1981); Holtwick et al., in Microbiology 147(Pt 2):337-44 (Febuary 2001).

[0236] Further examples of expression vectors that can be useful in *Pseudomonas* host cells include those listed in Table 16 as derived from the indicated replicons.

TABLE 16

SOME EXAMPLES OF USEFUL EXPRESSION VECTORS

Replicon	Vector(s)
$_P$PS10	$_P$CN39, $_P$CN51
RSF1010	$_P$KT261-3
	$_P$MMB66EH
	$_P$EB8
	$_P$PLGN1
	$_P$MYC1050
RK2/RP1	$_P$RK415
	$_P$JB653
$_P$RO1600	$_P$UCP
	$_P$BSP

[0237] The expression plasmid, RSF1010, is described, e.g., by F. Heffron et al., in Proc. Nat'l Acad. Sci. USA 72(9):3623-27 (September 1975), and by K. Nagahari & K. Sakaguchi, in J. Bact. 133(3):1527-29 (March 1978). Plasmid RSF1010O and derivatives thereof are particularly useful vectors in the present invention. Exemplary, useful derivatives of RSF1010, which are known in the art, include, e.g., pKT212, pKT214, pKT231 and related plasmids, and pMYC1050 and related plasmids (see, e.g., U.S. Pat, Nos. 5,527,883 and 5,840,554 to Thompson et al.), such as, e.g., pMYC1803. Plasmid pMYC1803 is derived from the RSF1010-based plasmid pTJS260 (see U.S. Pat. No. 5,169,760 to Wilcox), which carries a regulated tetracycline resistance marker and the replication and mobilization loci from the RSF1010 plasmid. Other exemplary useful vectors include those described in U.S. Pat. No. 4,680,264 to Puhler et al.

[0238] In a one embodiment, an expression plasmid is used as the expression vector. In another embodiment, RSF1010 or a derivative thereof is used as the expression vector. In still another embodiment, pMYC1050 or a derivative thereof, or pMYC1803 or a derivative thereof, is used as the expression vector.

VII. Expression of Recombinant Polypeptides in an Pseudomonad Host Cells

[0239] In one aspect of the present invention, processes of expressing recombinant polypeptides for use in improved protein production are provided.

[0240] In one embodiment, the process provides expression of a nucleic acid construct comprising nucleic acids encoding a) a recombinant polypeptide, and b) a prototrophy-restoring enzyme in a Pseudomonad that is auxotrophic for at least one metabolite. In an alternative embodiment, the Pseudomonad is auxotrophic for more than one metabolite. In one embodiment, the Pseudomonad is a *Pseudomonas fluorescens* cell. In a particular embodiment, a recombinant polypeptide is expressed in a Pseudomonad that is auxotrophic for a metabolite, or combination of metabolites, selected from the group consisting of a nitrogenous base compound and an amino acid. In a more particular embodiment, recombinant polypeptides are expressed in a Pseudomonad that is auxotrophic for a metabolite selected from the group consisting of uracil, proline, and thymidine. In another embodiment, the auxotrophy can be generated by the knock-out of the host pyrF, proC, or thyA gene, respectively. An alternative embodiment, recombinant polypeptides are expressed in an auxotrophic Pseudomonad cell that has been genetically modified through the insertion of a native *E.coli* lacI gene, $lacI^Q$ gene, or $lacI^{Q1}$ gene, other than as part of the PlacI-lacI-lacZYA operon, into the host cell's chromosome. In one particular embodiment, the vector containing the recombinant polypeptide expressed in the auxotrophic host cell comprises at least two lac operator sequences, or derivatives thereof. In still a further embodiment, the recombinant polypeptide is driven by a Plac family promoter.

[0241] In another embodiment, the process involves the use of Pseudomonad host cells that have been genetically modified to provide at least one copy of a LacI encoding gene inserted into the Pseudomonad host cell's genome, wherein the lacI encoding gene is other than as part of the PlacI-lacI-lacZYA operon. In one embodiment, the gene encoding the Lac repressor protein is identical to that of native *E.coli* lacI gene. In another embodiment, the gene encoding the Lac repressor protein is the $lacI^Q$ gene. In still another embodiment, the gene encoding the Lac repressor protein is the $lacI^{Q1}$ gene. In a particular embodiment, the Pseudomonad host cell is *Pseudomonas fluorescens*. In another embodiment, the Pseudomonad is further genetically modified to produce an auxotrophic cell. In another embodiment, the process produces recombinant polypeptide levels of at least about 3 g/L, 4 g/L, 5 g/L 6 g/L, 7 g/L, 8 g/L, 9 g/L or at least about 10 g/L. In another embodiment, the recombinant polypeptide is expressed in levels of between 3 g/L and 100 g/L.

[0242] The method generally includes:

[0243] a) providing a Pseudomonad host cell, preferably a *Pseudomonas fluorescens,* as described in the present invention,

[0244] b) transfecting the host cell with at least one nucleic acid expression vector comprising i) a target recombinant polypeptide of interest, and, in the case of the utilization of an auxotrophic host, ii) a gene encoding a prototrophy enabling enzyme that, when expressed, overcomes the auxotrophy of the host cell;

[0245] c) growing the host cell in a growth medium that provides a selection pressure effective for maintaining the nucleic acid expression vector containing the recombinant polypeptide of interest in the host cell; and

[0246] d) expressing the target recombinant polypeptide of interest.

[0247] The method can further comprise transfecting the host cell with at least once nucleic acid expression vector further comprising iii) a Plac family promoter, and optionally iv) more than one lac operator sequences. In one embodiment, at least one lac operator sequence may be a lac_Oid sequence. Preferably, the expression system is capable of expressing the target polypeptide at a total productivity of polypeptide of at least 1 g/L to at least 80 g/L. In a particular embodiment, the recombinant polypeptide is expressed at a level of at least 3 g/L, 4g/L, 5g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, 10 g/L, 12 g/L, 15 g/L, 20 g/L, 25 gL, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 60 g/L, 70 g/L, or at least 80 g/L. In a particular embodiment, a lac or tac family promoter is utilized in the present invention, including Plac, Ptac, Ptrc, PtacII, PlacUV5, 1pp-PlacUV5, 1pp-lac, nprM-lac, T71ac, T51ac, T31ac, and Pmac.

[0248] In one embodiment, at least one recombinant polypeptide can be expressed in a Pseudomonad cell that is auxotrophic for one metabolite, wherein the auxotrophy serves as a selection marker for the maintenance of the nucleic acid expression vector encoding the polypeptide of interest and the prototrophy-enabling enzyme. Alternatively, more than one recombinant polypeptide can be expressed in a Pseudomonad cell that is auxotrophic for one metabolite, wherein the nucleic acids encoding the recombinant polypeptides can be contained on the same vector, or alternatively, on multiple vectors.

[0249] In yet another embodiment, more than one expression vector encoding different target polypeptides can be maintained in a Pseudomonad host cell auxotrophic for at least one metabolite, wherein one expression vector contains a nucleic acid encoding a prototrophic-enabling enzyme and a first target polypeptide of interest, and a second expression vector contains a nucleic acid encoding an alternative, non-auxotrophic selection marker and a second polypeptide of interest.

[0250] In another embodiment, at least one recombinant polypeptide can be expressed in a Pseudomonad cell that is auxotrophic for more than one metabolite, wherein the multiple auxotrophies serve as selection markers for the maintenance of nucleic acid expression vectors. For example, an expression vector may be utilized in which a first and second prototrophy-enabling selection marker gene are present. If both marker genes are located on the same DNA construct, the host cell containing the construct may be maintained under either or both of the conditions in which host cell survival requires the presence of the selection

marker gene(s). When only one of the marker-gene dependent survival conditions is present, the corresponding marker gene must be expressed, and the other marker gene(s) can then be either active or inactive, though all necessary nutrients for which the cell remains auxotrophic will still be supplied by the medium. This permits the same target gene, or the same set of covalently linked target genes, encoding the desired transgenic product(s) and/or desired transgenic activity(ies), to be maintained in the host cell continuously as the host cell is transitioned between or among different conditions. If each of the two selection marker genes is located on a different DNA construct, then, in order to maintain both of the DNA constructs in the host cell, both of the marker-gene dependent survival conditions are present, and both of the corresponding marker gene must be expressed. This permits more than one non-covalently linked target gene or set of target gene(s) to be separately maintained in the host cell. The coding sequence of each of the chosen selection marker genes independently can be operatively attached to either a constitutive or a regulated promoter.

[0251] Dual-target-gene examples of such a multi-target-gene system include, but are not limited to: (1) systems in which the expression product of one of the target genes interacts with the other target gene itself; (2) systems in which the expression product of one of the target genes interacts with the other target gene's expression product, e.g., a protein and its binding protein or the α- and β-polypeptides of an αn-βn protein; (3) systems in which the two expression products of the two genes both interact with a third component, e.g., a third component present in the host cell; (4) systems in which the two expression products of the two genes both participate in a common biocatalytic pathway; and (5) systems in which the two expression products of the two genes function independently of one another, e.g., a bi-clonal antibody expression system.

[0252] In one example of a dual-target-gene system of the above-listed type (1), a first target gene can encode a desired target protein, wherein the first target gene is under the control of a regulated promoter; the second target gene may then encode a protein involved in regulating the promoter of the first target gene, e.g., the second target gene may encode the first target gene's promoter activator or repressor protein. In an example in which the second gene encodes a promoter regulatory protein for the first gene, the coding sequence of the second gene can be under the control of a constitutive promoter. In one embodiment, the second gene will be part of a separate DNA construct that is a maintained in the cell as a high-copy-number construct with a copy number of at least 10, 20, 30, 40, 50, or more than 50 copies being maintained in the host cell.

[0253] In another embodiment, the present invention provides the use of more than one lacO sequence on an expression vector in the production of recombinant polypeptides in Pseudomonads, particularly in *Pseudomonas fluorescens*

[0254] In another aspect, the present invention provides a method of producing a recombinant polypeptide comprising transforming a bacterial host cell that is a member of the Pseudomonads and closely related bacteria having at least one chromosomally inserted copy of a Lac repressor protein encoding a lacI transgene, or derivative thereof such as $lacI^{Q1}$ or $lacI^{Q1,}$ which transgene is other than part of a whole or truncated structural gene containing PlacI-lacI-lacZYA construct with a nucleic acid construct encoding at least one target recombinant polypeptide. The nucleic acid encoding at least one target recombinant polypeptide can be operably linked to a Plac family promoter, in which all of the Plac family promoters present in the host cell are regulated by Lac repressor proteins expressed solely from the lacI transgene inserted in the chromosome. Optionally, the expression system is capable of expressing the target polypeptide at a total productivity of at least 3 g/L to at least 10 g/L. Preferably, the expression system is capable of expressing the target polypeptide at a total productivity of polypeptide of at least 3 g/L, 4g/L, 5g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L, or at least 10 g/L.

[0255] In one embodiment, the present invention provides a method of expressing recombinant polypeptides in an expression system utilizing auxotrophic Pseudomonads or related bacteria that have been further genetically modified to provide at least one copy of a LacI encoding gene inserted into the cell's genome, other than as part of the PlacI-lacI-lacZYA operon. In a particular embodiment, a recombinant polypeptide is expressed in an auxotrophic *Pseudomonas fluorescens* host cell containing a lacI transgene insert. In another particular embodiment, a recombinant polypeptide is expressed in an auxotrophic *Pseudomonas fluorescens* host cell containing a $lacI^{Q1}$ transgene insert. In still another particular embodiment, a recombinant polypeptide is expressed in an auxotrophic *Pseudomonas fluorescens* host cell containing a $lacI^{Q1}$ transgene insert. The *Pseudomonas fluorescens* host can be auxotrophic for a biochemical required by the cell for survival. In a particular embodiment, the *Pseudomonas fluorescens* cell is auxotrophic for a nitrogenous base. In a particular embodiment, the *Pseudomonas fluorescens* is auxotrophic for a nitrogenous base selected from the group consisting of thymine and uracil. In a particularly particular embodiment, the *Pseudomonas fluorescens* host cell's auxotrophy is induced by a genetic modification to a pyrF or thyA gene rendering the associated encoded product non-functional. In an alternative embodiment, the *Pseudomonas fluorescens* cell is auxotrophic for an amino acid. In a particular embodiment, the *Pseudomonas fluorescens* is auxotrophic for the amino acid proline. In a particularly particular embodiment, the *Pseudomonas fluorescens* host cell's auxotrophy is induced by a genetic modification to a proC gene rendering the associated encoded product non-functional.

[0256] Transformation

[0257] Transformation of the Pseudomonad host cells with the vector(s) may be performed using any transformation methodology known in the art, and the bacterial host cells may be transformed as intact cells or as protoplasts (i.e. including cytoplasts). Exemplary transformation methodologies include poration methodologies, e.g., electroporation, protoplast fusion, bacterial conjugation, and divalent cation treatment, e.g., calcium chloride treatment or CaCl/Mg^{2}+ treatment, or other well known methods in the art. See, e.g., Morrison, J. Bact., 132:349-351 (1977); Clark-Curtiss & Curtiss, Methods in Enzymology, 101:347-362 (Wu et al., eds, 1983), Sambrook et al., Molecular Cloning, A Laboratory Manual (2nd ed. 1989); Kriegler, Gene Trans-

fer and Expression: A Laboratory Manual (1990); and Current Protocols in Molecular Biology (Ausubel et al., eds., 1994)).

[0258] Selection

[0259] Preferably, cells that are not successfully transformed are selected against following transformation, and continuously during the fermentation. The selection marker can be an auxotrophic selection marker or a traditional antibiotic selection marker. When the cell is auxotrophic for multiple nutrient compounds, the auxotrophic cell can be grown on medium supplemented with all of those nutrient compounds until transformed with the prototrophy-restoring vector. Where the host cell is or has been made defective for multiple biosynthetic activities, the prototrophy-restorative marker system(s) can be selected to restore one or more or all of the biosynthetic activities, with the remainder being compensated for by continuing to provide, in the medium, the still-lacking nutrients. In selection marker systems in which more than one biosynthetic activity, and/or more than one prototrophy, is restored, the plurality of selection marker genes may be expressed together on one vector or may be co-expressed separately on different vectors. Even where a single metabolite is the target of the selection marker system, multiple biosynthetic activities may be involved in the selection marker system. For example, two or more genes encoding activities from the same anabolic pathway may be expressed together on one vector or may be co-expressed separately on different vectors, in order to restore prototrophy in regard to biosynthesis of the compound that is the product of the pathway.

[0260] Where the selection marker is an antibiotic resistance gene, the associated antibiotic can be added to the medium to select against non transformed and revertant cells, as well known in the art.

[0261] Fermentation

[0262] As used herein, the term "fermentation" includes both embodiments in which literal fermentation is employed and embodiments in which other, non-fermentative culture modes are employed. Fermentation may be performed at any scale. In one embodiment, the fermentation medium may be selected from among rich media, minimal media, a mineral salts media; a rich medium may be used, but is preferably avoided. In another embodiment either a minimal medium or a mineral salts medium is selected. In still another embodiment, a minimal medium is selected. In yet another embodiment, a mineral salts medium is selected. Mineral salts media are particularly particular.

[0263] Prior to transformation of the host cell with a nucleic acid construct encoding a prototrophic enabling enzyme, the host cell can be maintained in a media comprising a supplemental metabolite, or analogue thereof, that complements the auxotrophy. Following transformation, the host cell can be grown in a media that is lacking the complementary metabolite that the host cell is auxotrophic for. In this way, host cells that do not contain the selection marker enabling prototrophy are selected against. Likewise cells expressing recombinant proteins from expression vectors containing an antibiotic resistance selection marker gene can be maintained prior to transformation on a medium lacking the associated antibiotic used for selection. After transformation and during the fermentation, an antibiotic can be added to the medium, at concentrations known in the art, to select against non-transformed and revertant cells.

[0264] Mineral salts media consists of mineral salts and a carbon source such as, e.g., glucose, sucrose, or glycerol. Examples of mineral salts media include, e.g., M9 medium, *Pseudomonas* medium (ATCC 179), Davis and Mingioli medium (see, B D Davis & E S Mingioli, in J. Bact. 60:17-28 (1950)). The mineral salts used to make mineral salts media include those selected from among, e.g., potassium phosphates, ammonium sulfate or chloride, magnesium sulfate or chloride, and trace minerals such as calcium chloride, borate, and sulfates of iron, copper, manganese, and zinc. No organic nitrogen source, such as peptone, tryptone, amino acids, or a yeast extract, is included in a mineral salts medium. Instead, an inorganic nitrogen source is used and this may be selected from among, e.g., ammonium salts, aqueous ammonia, and gaseous ammonia. A particular mineral salts medium will contain glucose as the carbon source. In comparison to mineral salts media, minimal media can also contain mineral salts and a carbon source, but can be supplemented with, e.g., low levels of amino acids, vitamins, peptones, or other ingredients, though these are added at very minimal levels.

[0265] In one embodiment, media can be prepared using the components listed in Table 16 below. The components can be added in the following order: first $(NH_4)HPO_4$, KH_2PO_4 and citric acid can be dissolved in approximately 30 liters of distilled water; then a solution of trace elements can be added, followed by the addition of an antifoam agent, such as Ucolub N 115. Then, after heat sterilization (such as at approximately 121° C.), sterile solutions of glucose $MgSO_4$ and thiamine-HCL can be added. Control of pH at approximately 6.8 can be achieved using aqueous ammonia. Sterile distilled water can then be added to adjust the initial volume to 371 minus the glycerol stock (123 mL). The chemicals are commercially available from various suppliers, such as Merck. This media can allow for high cell density cultivation (HCDC) for growth of Pseudomonas species and related bacteria. The HCDC can start as a batch process which is followed by two-phase fed-batch cultivation. After unlimited growth in the batch part, growth can be controlled at a reduced specific growth rate over a period of 3 doubling times in which the biomass concentration can increased several fold. Further details of such cultivation procedures is described by Riesenberg, D.; Schulz, V.; Knorre, W. A.; Pohl, H. D.; Korz, D.; Sanders, E. A.; Ross, A.; Deckwer, W. D. (1991) "High cell density cultivation of *Escherichia* coli at controlled specific growth rate" J Biotechnol: 20(1) 17-27.

[0266] The expression system according to the present invention can be cultured in any fermentation format. For example, batch, fed-batch, semi-continuous, and continuous fermentation modes may be employed herein.

[0267] The expression systems according to the present invention are useful for transgene expression at any scale (i.e. volume) of fermentation. Thus, e.g., microliter-scale, centiliter scale, and deciliter scale fermentation volumes may be used; and 1 Liter scale and larger fermentation volumes can be used. In one embodiment, the fermentation volume will be at or above 1 Liter. In another embodiment, the fermentation volume will be at or above 5 Liters, 10 Liters, 15 Liters, 20 Liters, 25 Liters, 50 Liters, 75 Liters,

100 Liters, 200 Liters, 50 Liters, 1,000 Liters, 2,000 Liters, 5,000 Liters, 10,000 Liters or 50,000 Liters.

[0268] In the present invention, growth, culturing, and/or fermentation of the transformed host cells is performed within a temperature range permitting survival of the host cells, preferably a temperature within the range of about 4° C. to about 55° C., inclusive.

[0269] Cell Density

[0270] An additional advantage in using *Pseudomonas fluorescens* in expressing recombinant proteins includes the ability of *Pseudomonas fluorescens* to be grown in high cell densities compared to *E.coli* or other bacterial expression systems. To this end, *Pseudomonas fluorescens* expressions systems according to the present invention can provide a cell density of about 20 g/L or more. The *Pseudomonas fluorescens* expressions systems according to the present invention can likewise provide a cell density of at least about 70 g/L, as stated in terms of biomass per volume, the biomass being measured as dry cell weight.

[0271] In one embodiment, the cell density will be at least 20 g/L. In another embodiment, the cell density will be at least 25 g/L, 30 g/L, 35 g/L, 40 g/L, 45 g/L, 50 g/L, 60 g/L, 70 g/L 80 g/L, 90 g/L., 100 g/L, 110 g/L, 120 g/L, 130 g/L, 140 g/L, or at least 150 g/L.

[0272] In another embodiments, the cell density at induction will be between 20 g/L and 150 g/L;, 20 g/L and 120 g/L; 20 g/L and 80 g/L; 25 g/L and 80 g/L; 30 g/L and 80 g/L; 35 g/L and 80 g/L; 40 g/L and 80 g/L; 45 g/L and 80 g/L; 50 g/L and 80 g/L; 50 g/L and 75 g/L; 50 g/L and 70 g/L; 40 g/L and 80 g/L.

[0273] Expression Levels of Recombinant Protein

[0274] The expression systems according to the present invention can express transgenic polypeptides at a level at between 5% and 80% total cell protein (%tcp). In one embodiment, the expression level will be at or above 5%, 8%, 10%, 12%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%,70%, 75%, or 80% tcp.

[0275] Isolation and Purification

[0276] The recombinant proteins produced according to this invention may be isolated and purified to substantial purity by standard techniques well known in the art, including, but not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, nickel chromatography, hydroxylapatite chromatography, reverse phase chromatography, lectin chromatography, preparative electrophoresis, detergent solubilization, selective precipitation with such substances as column chromatography, immunopurification methods, and others. For example, proteins having established molecular adhesion properties can be reversibly fused a ligand. With the appropriate ligand, the protein can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. In addition, protein can be purified using immunoaffinity columns or Ni-NTA columns. General techniques are further described in, for example, R. Scopes, Protein Purification: Principles and Practice, Springer-Verlag: N.Y. (1982); Deutscher, Guide to Protein Purification, Academic Press (1990); U.S. Pat. No. 4,511,503; S. Roe, Protein Purification Techniques: A Practical Approach (Practical Approach Series), Oxford Press (2001); D. Bollag, et al., Protein Methods, Wiley-Lisa, Inc. (1996); A K Patra et al., Protein Expr Purif, 18(2): p/ 182-92 (2000); and R. Mukhija, et al., Gene 165(2): p. 303-6 (1995). See also, for example, Ausubel, et al. (1987 and periodic supplements); Deutscher (1990) "Guide to Protein Purification," Methods in Enzymology vol. 182, and other volumes in this series; Coligan, et al. (1996 and periodic Supplements) Current Protocols in Protein Science Wiley/Greene, NY; and manufacturer's literature on use of protein purification products, e.g., Pharmacia, Piscataway, N.J., or Bio-Rad, Richmond, Calif. Combination with recombinant techniques allow fusion to appropriate segments, e.g., to a FLAG sequence or an equivalent which can be fused via a protease-removable sequence. See also, for example., Hochuli (1989) Chemische Industrie 12:69-70; Hochuli (1990) "Purification of Recombinant Proteins with Metal Chelate Absorbent" in Setlow (ed.) Genetic Engineering, Principle and Methods 12:87-98, Plenum Press, NY; and Crowe, et al. (1992) QIAexpress: The High Level Expression & Protein Purification System QUIAGEN, Inc., Chatsworth, Calif.

[0277] Detection of the expressed protein is achieved by methods known in the art and includes, for example, radioimmunoassays, Western blotting techniques or immunoprecipitation.

[0278] The recombinantly produced and expressed enzyme can be recovered and purified from the recombinant cell cultures by numerous methods, for example, high performance liquid chromatography (HPLC) can be employed for final purification steps, as necessary.

[0279] Certain proteins expressed in this invention may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purification of proteins from inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of the host cells, e.g., by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM MgCl.sub.2, 1 mM DTT, 0.1 mM ATP, and 1 mM PMSF. The cell suspension is typically lysed using 2-3 passages through a French Press. The cell suspension can also be homogenized using a Polytron (Brinknan Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (see, e.g., Sambrook et al., supra; Ausubel et al., supra).

[0280] If necessary, the inclusion bodies can be solubilized, and the lysed cell suspension typically can be centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of immunologically and/or biologically active protein. Other suitable buffers are known to those skilled in the art.

[0281] Alternatively, it is possible to purify the recombinant proteins or peptides from the host periplasm. After lysis

of the host cell, when the recombinant protein is exported into the periplasm of the host cell, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to those skilled in the art. To isolate recombinant proteins from the periplasm, for example, the bacterial cells can be centrifuged to form a pellet. The pellet can be resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria can be centrifuged and the pellet can be resuspended in ice-cold 5 mM MgSO.sub.4 and kept in an ice bath for approximately 10 minutes. The cell suspension can be centrifuged and the supernatant decanted and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

[0282] An initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. One such example can be ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

[0283] The molecular weight of a recombinant protein can be used to isolated it from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture can be ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration can then be ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

[0284] Recombinant proteins can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

[0285] Renaturation and Refolding

[0286] Insoluble protein can be renatured or refolded to generate secondary and tertiary protein structure conformation. Protein refolding steps can be used, as necessary, in completing configuration of the recombinant product. Refolding and renaturation can be accomplished using an agent that is known in the art to promote dissociation/association of proteins. For example, the protein can be incubated with dithiothreitol followed by incubation with oxidized glutathione disodium salt followed by incubation with a buffer containing a refolding agent such as urea.

[0287] Recombinant protein can also be renatured, for example, by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be refolded while immobilized on a column, such as the Ni NTA column by using a linear 6M-7M urea gradient in 500 mM NaCl, 20% glycerol, 20 mM Tris/HCl pH 7.4, containing protease inhibitors. The renaturation can be performed over a period of 1.5 hours or more. After renaturation the proteins can be eluted by the addition of 250 mM immidazole. Immidazole can be removed by a final dialyzing step against PBS or 50 mM sodium acetate pH 6 buffer plus 200 mM NaCl. The purified protein can be stored at 4.degree. C. or frozen at -80.degree. C.

[0288] Other methods include, for example, those that may be described in M H Lee et al., Protein Expr. Purif., 25(1): p. 166-73 (2002), W. K. Cho et al., J. Biotechnology, 77(2-3): p. 169-78 (2000), Ausubel, et al. (1987 and periodic supplements), Deutscher (1990) "Guide to Protein Purification," Methods in Enzymology vol. 182, and other volumes in this series, Coligan, et al. (1996 and periodic Supplements) Current Protocols in Protein Science Wiley/Greene, NY, S. Roe, Protein Purification Techniques: A Practical Approach (Practical Approach Series), Oxford Press (2001); D. Bollag, et al., Protein Methods, Wiley-Lisa, Inc. (1996).

VI. Recombinant Polypeptides

[0289] The present invention provides improved protein production in bacterial expression systems. Examples of recombinant polypeptides that can be used in the present invention include polypeptides derived from prokaryotic and eukaryotic organisms. Such organisms include organisms from the domain Archea, Bacteria, Eukarya, including organisms from the Kingdom Protista, Fungi, Plantae, and Animalia.

[0290] Types of proteins that can be utilized in the present invention include non-limiting examples such as enzymes, which are responsible for catalyzing the thousands of chemical reactions of the living cell; keratin, elastin, and collagen, which are important types of structural, or support, proteins; hemoglobin and other gas transport proteins; ovalbumin, casein, and other nutrient molecules; antibodies, which are molecules of the immune system; protein hormones, which regulate metabolism; and proteins that perform mechanical work, such as actin and myosin, the contractile muscle proteins.

[0291] Other specific non-limiting polypeptides include molecules such as, e.g., renin, a growth hormone, including human growth hormone; bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha. 1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; thrombopoietin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC,

factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial naturietic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; a serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; Dnase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-.beta.; cardiotrophins (cardiac hypertrophy factor) such as cardiotrophin-1 (CT-1); platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-.beta.1, TGF-.beta.2, TGF-.beta.3, TGF-.beta.4, or TGF-.beta.5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD-3, CD-4, CD-8, and CD-19; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; anti-HER-2 antibody; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; antibodies; and fragments of any of the above-listed polypeptides.

[0292] The recombinant peptides to be expressed by according to the present invention can be expressed from polynucleotides in which the target polypeptide coding sequence is operably attached to transcription and translation regulatory elements to form a functional gene from which the host cell can express the protein or peptide. The coding sequence can be a native coding sequence for the target polypeptide, if available, but will more preferably be a coding sequence that has been selected, improved, or optimized for use in the selected expression host cell: for example, by synthesizing the gene to reflect the codon use bias of a *Pseudomonas* species such as *Pseudomonas fluorescens*. The gene(s) that result will have been constructed within or will be inserted into one or more vector, which will then be transformed into the expression host cell. Nucleic acid or a polynucleotide said to be provided in an "expressible form" means nucleic acid or a polynucleotide that contains at least one gene that can be expressed by the selected bacterial expression host cell.

[0293] Extensive sequence information required for molecular genetics and genetic engineering techniques is widely publicly available. Access to complete nucleotide sequences of mammalian, as well as human, genes, cDNA sequences, amino acid sequences and genomes can be obtained from GenBank at the URL address http://www.ncbi.nlm.nih.gov/Entrez. Additional information can also be obtained from GeneCards, an electronic encyclopedia integrating information about genes and their products and biomedical applications from the Weizmann Institute of Science Genome and Bioinformatics (http:/Ibioinformatics.weizmann.ac.il/cards/), nucleotide sequence information can be also obtained from the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/) or the DNA Databank or Japan (DDBJ, http://www.ddbi.nig.ac.jp/; additional sites for information on amino acid sequences include Georgetown's protein information resource website (http://www-nbrf.georgetown.edu/pir/) and Swiss-Prot (http://au.expasy.org/sprot/sprot-top.html).

EXAMPLES

Example 1

Construction of a pyrF Selection Marker System in a *P. fluorescens* Host Cell Expression System

[0294] Reagents were acquired from Sigma-Aldrich (St. Louis Mo.) unless otherwise noted. LB is 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCI in a gelatin capsule (BIO 101). When required, uracil (from BIO101, Carlsbad Calif.) or L-proline was added to a final concentration of 250 ug/mL, and tetracycline was added to 15 ug/mL. LB/5-FOA plates contain LB with 250 mM uracil and 0.5 mg/mL 5-fluoroorotic acid (5-FOA). M9 media consists of 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 10 mM $MgSO_4$, 1× HoLe Trace Element Solution, pH7. Glucose was added to a final concentration of 1%. The 1000× HoLe Trace Element Solution is 2.85 g/L H_3BO_3, 1.8 g/L $MnCl_2$. 4H2O, 1.77 g/L sodium tartrate, 1.36 g/L $FeSO_4$. 7H2O, 0.04 g/L $CoCl_2$. $6H_2O$, 0.027 g/L $CuCl_2$. $2H_2O$, 0.025 g/L Na_2MoO_4. $2H_2O$, 0.02 g/L $ZnCl_2$.

Oligonucleotides Used Herein

[0295]

MB214pyrF1 (NotI site in bold)
5'-**GCGGCCGC**TTTGGCGCTTCGTTTACAGG-3' (SEQ ID NO: 14)

MB214pyrR1 (PvuI site in bold;
KpnI site in underlined bold)
5'-**CGATCGG**<u>**GTACC**</u>TGTCGAAGGGCTGGAGACA (SEQ ID NO: 15)
T-3'

pyrFPstF (PstI site in bold)
5'-AA**CTGCAG**GATCAGTTGCGGAGCCTTGG-3' (SEQ ID NO: 16)

pyrFoverlap
5'-TGCTCACTCTAAAAATCTGGAATGGGCTCTC (SEQ ID NO: 17)
AGGC-3'

pyrFXbaR2 (XbaI site in bold)
5'-GC**TCTAGA**TGCGTGGCTGGATGAATGAA-3' (SEQ ID NO: 18)

pyrana1F
5'-GGCGTCGAACAGGTAGCCTT-3' (SEQ ID NO: 19)

pyrana1R
5'-CTCGCCTCCTGCCACATCAA-3' (SEQ ID NO: 20)

M13F(-40)
5'-CAGGGTTTTCCCAGTCACGA-3' (SEQ ID NO: 21)

Cloning of a pyrF *gene* from *P. fluorescens*

[0296] The pyrF gene was cloned from *P. fluorescens* by polymerase chain reaction (PCR) amplification, using primers MB214pyrF1 and MB214pyrR1 that bind 297 bp upstream from the pyrF gene start codon and 212 bp

downstream of its stop codon, respectively. Restriction sites were included at the 5' ends of the primers to facilitate further cloning reactions The amplified region upstream of the pyrF open reading frame (ORF) was estimated as long enough to include the native promoter upstream of pyrF. A strong stem-loop structure at 14-117 bp downstream of the pyrF ORF, which may be a transcription terminator, was also included in the downstream flanking region.

[0297] To PCR-amplify the pyrF gene, the high-fidelity PROOFSTART DNA polymerase was mixed in a 50 uL reaction volume containing buffer provided by the manufacturer (Qiagen, Valencia Calif.) 0.3 mM dNTPs (Promega, Madison, Wis.), 1 uM each of MB214pyrF1 and MB214pyrR1 primers, and about 0.3 μg of genomic DNA from *P. fluorescens* MB214. The amplification conditions were 5 min at 95° C., followed by 35 cycles of a 30 sec denaturation at 94° C., 30 sec annealing at 57° C., and a 2 min extension at 72° C., followed by a final step a 72° C. for 10 min. The reaction was separated on a 1% gel of SEAKEM GTG agarose (from BioWhittaker Molecular Applications , Rockland Me.). The expected 1.2 kb band was excised from the gel and purified by extraction on a ULTRAFREE-DA centrifugal gel nebulizer from Millipore (Bedford Mass.) column and de-salted into Tris-HCI buffer with a MICRoBIoSPIN 6 P-6 polyacrylamide spin column (from Bio-Rad, Hercules Calif.).

[0298] The cloned gene contained a single ORF, encoding orotidine 5' phosphate decarboxylase. The identity of the gene was further confirmed as pyrF by its high similarity (P-value of 3.3×10^{-78}) along the entire length of the gene (209 out of 232 residues) to the pyrF gene from *P. aeruginosa,* which had been previously reported (Strych et al., 1994). The *P. fluorescens* strain used was found to contain no other copies of anypyrF genes.

[0299] Sequencing was performed by The Dow Chemical Company. The pyrF sequence is presented within SEQ ID NO:1.

Construction of a pyrF(−) *P. fluorescens*

[0300] To construct a pyrF(−) *P. fluorescens* , the cell's genomic pyrF gene was altered by deleting of the ORF between and including the gene's start and stop codons. The deletion was made by fusing in vitro the upstream and downstream regions flanking the pyrF region on a nonreplicating plasmid, then using allele exchange, i.e. homologous recombination, to replace the endogenous pyrF gene in MB101 with the deletion allele.

[0301] To construct the fusion of the flanking regions, the "Megaprimer" method (Barik 1997) was used, whereby the region upstream and then downstream of the desired deletion were subsequently amplified by PCR using an overlapping primer with homology on both sides of the desired deletion, so that the flanking regions become linked, leaving out the pyrF ORF. The upstream region was amplified from MB214 genomic DNA using the Proofstart polymerase (Qiagen) as described above, with the primers pyrFPstF and pyrFoverlap, and an extension time of 1 minute. After gel purification using binding to glass milk (GENECLEAN Spin Kit from Bio101, Carlsbad, Calif., USA), the 1 kB product was used as the "Megaprimer" for the second amplification.

[0302] Because there was difficulty amplifying the desired product in this second step, a template containing the genomic pyrF region was made by PCR amplification in order to increase the template quantity. HOTSTARTAQ DNA polymerase (from Qiagen, Valencia Calif.) was used with *P. fluorescens* genomic DNA and the pyrFPstF and pyrFXbaR2 primers. The Megaprimer and the pyrFXbaR2 primer were then used with this template and HOTSTARTAQ polymerase, to amplify the deletion product by PCR, using amplification conditions of 15 min at 95° C., followed by 30 cycles of a 30 sec denaturation at 94° C., 30 sec annealing at 59° C., and a 2 min extension at 72° C., followed by a final step at 72° C. for 3 min. The expected 2 kB band was separated from a number of other products by gel electrophoresis, and then gel purified as above and cloned into plasmid pCR2.1Topo (from Invitrogen, Carlsbad CA) according to instructions from the manufacturer, to form pDOW1215-7 Sequencing the PCR-amplified region of pDOW1215-7 showed that there were 3 mutations introduced by the amplification process; all three changes were within 112 bp downstream of the stop codon for pyrF. Sequencing through this area was difficult, because the process of the reaction stopped in this area. Analysis by M-FOLD (GCG) of the secondary structure of RNA that would be encoded by this area showed the presence of a very stable stem-loop structure and a run of uridine residues that is characteristic of a rho-independent transcription terminator. None of the mutations occurred in the open reading frame. pDOW 1215-7 was used to delete the chromosomal pyrF gene in MB 101. To do this, first, electrocompetent *P. fluorescens* cells made according to the procedure of Artiguenave et al. (1997), were transformed with 0.5 μg of the purified plasmid. Transformants were selected by plating on LB medium with kanamycin at 50 μg/mL. This plasmid cannot replicate in *P. fluorescens* , therefore kanamycin resistant colonies result from the plasmid integrating into the chromosome. The site of integration of the plasmid was analyzed by PCR using the HOTSTARTAQ polymerase and primers pryanalF and M13F(−40), annealing at 57° C. and with an extension time of 4 min. One out of the 10 isolates (MB101::pDOW1215-7#2) contained an insertion of pDOW1215-7 into the downstream region (2.8 kB analytical product) and in the other nine were in the upstream region (2.1 kb analytical product).

[0303] Second, to identify strains that had lost the integrated plasmid by recombination between the homologous regions the following analytical PCR procedure was used: MB1010::pDOW1215-7#2 was inoculated from a single colony into LB supplemented with 250 mM uracil, grown overnight, and then plated onto LB-uracil and 500 μg/mL 5-fluoroorotic acid (5-FOA—Zymo Research, Orange Calif.). Eight colonies were analyzed by PCR with HOTSTARTAQ and primers pyranalF and pyranalR, annealing at 57° C. and extending for 4 min. The expected size of the amplified product from the parent MB 101 was 3.2 kB, or if the pyrF gene was deleted, then 2.5 kB. Each of the colonies gave rise to the 2.5 kB band expected from a deletion of pyrF. The first three isolates were purified and named PFG116, PFG117, and PFG118 (also known as DC36). The three isolates exhibit the phenotype expected from a pyrF deletion, i.e. they are sensitive to kanamycin, uracil is required for growth, and they are resistant to 5-FOA. The DNA sequence of PFG118 was identical to that of the amplified regions in pDOW1215-7; i.e. the three mutations in the stem-loop structure immediately downstream from pyrF were incorporated into the PFG 118 genome, along with the pyrF deletion.

Use of the pyrF Gene as a Selection Marker in *P. fluorescens* Expression System

[0304] The ability of the pyrF gene to act as a selectable marker was tested by cloning it into a pMYC expression plasmid containing both an existing tetracycline resistance marker and the target enzyme coding sequence under the control of the tac promoter. For this, the plasmid pMYC5088 was digested at 37° C. for 2 hr with SnaBI in a 50 uL reaction using NEB Buffer 4 and 0.1 mg/mL of bovine serum albumen (BSA) (from New England Biolabs, Beverly Mass.). The reaction mixture was then treated at 70° C. for 20 min to inactivate the enzyme, then gel-purified as described above. 60 ng of the SnaBI-digested pMYC5088 was ligated to 50 ng of the MB214pyrF1- MB214pyrR1 PCR product using the FAST-LINK DNA Ligation Kit (Epicentre Technologies, Madison Wis.). After 1 hr at 25° C., the reaction was stopped by treating the mixture at 70° C. for 20 min. The result was then transformed into chemically-competent JM109 *E.coli* cells (Promega Corp., Madison Wis.) using conditions recommended by the manufacturer.

[0305] Transformants were selected on LB medium containing tetracycline at 15 μg/mL. Plasmid DNA was prepared from 12 isolates using the QiaPrep Spin Miniprep Kit (Qiagen, Valencia Calif.) and screened with NotI and EcoRI, which indicated that one isolate contained the desired clone, pDOW1249-2 (**FIG. 2**). The plasmid pDOW1249-2 was transformed into pyrF(−) *P. fluorescens* containing a pCN plasmid containing a lacI repressor expression cassette and a kanamycin resistance marker gene. Isolates were tested in shake flasks and in 20-L fernentors.

[0306] Isolates were grown in minimal salts medium and kanamycin, but no tetracycline, so that the only selective pressure for the pDOW1249-2 plasmid was provided by the ability of the pyrF gene on the plasmid to complement the pyrF deletion in the chromosome. As determined by SDS-PAGE analysis, the amount of target protein produced by the new strain in the shake flask test was similar to that of the control strain, a genomically pyrF(+) *P. fluorescens* control system containing the same two plasmids, but for the absence of the pyrF gene in pDOW1249-2, and grown on the same medium but further supplemented with tetracycline in order to maintain the plasmid (data not shown). Two strains were chosen for further analysis at the 20-L scale, based on the amount of target protein seen on the SDS-PAGE gel and OD_{575} values in shake flasks. Both strains showed a level of accumulation of target protein within the normal range observed for the control strain (**FIG. 1**).

Example 2

Construction of a pyrF—proC Dual Auxotrophic Selection Marker System in a *P. fluorescens* Host Cell Expression System

[0307] Oligonucleotides Used Herein

proC1
5'-ATAT**GAGCTC**CGACCTTGAGTCGGCCATTG-3' (SEQ ID NO: 22)

proC2
5'-ATAT**GAGCTC**GGATCCAGTACGATCAGCAGGTACAG-3' (SEQ ID NO: 23)

-continued

proC3
5'-AGCAACACGCGTATTGCCTT-3' (SEQ ID NO: 24)

proC5
5'-GCCCTTGAGTTGGCACTTCATCG-3' (SEQ ID NO: 25)

proC6
5'-GATAAACGCGAAGATCGGCGAGATA-3' (SEQ ID NO: 26)

proC7
5'-CCGAGCATGTTTGATTAGACAGGTCCTTATTTCGA-3' (SEQ ID NO: 27)

proC8
5'-TGCAACGTGACGCAAGCAGCATCCA-3' (SEQ ID NO: 28)

proC9
5'-GGAACGATCAGCACAAGCCATGCTA-3' (SEQ ID NO: 29)

genF2
5'-ATAT**GAGCTC**TGCCGTGATCGAAATCCAGA-3' (SEQ ID NO: 30)

genR2
5'-ATAT**GGATCC**CGGCGTTGTGACAATTTACC-3' (SEQ ID NO: 31)

XbaNotDraU2 linker
5'-TCTAGAGCGGCCGCGTT-3' (SEQ ID NO: 32)

XbaNotDraL linker
5'-GCGGCCGCTCTAGAAAC-3' (SEQ ID NO: 33)

Cloning of proC from *P. fluorescens* and Formation of a pCN Expression Plasmid Containing proC

[0308] Replacing Antibiotic Resistant Gene in pCN51lacI with proC

[0309] The proC ORF and about 100 bp of adjacent upstream and downstream sequence was amplified from MB101 genomic DNA using proC1 and proC2, an annealing temperature of 56° C. and a 1 min extension. After gel purification of the 1 kB product and digestion with SacI, the fragment was cloned into SacI-digested pDOW1243 (a plasmid derived from pCN51lacI by addition of a polylinker and replacement of kanR with the gentamycin resistance gene), to create pDOW1264-2. This plasmid was tested in the proC(−) mutant strain PFG932 for its ability to regulate amylase synthesis from pDOW1249-2. Expressed target enzyme production levels at the 20-L scale was similar to that of the dual-antibiotic-resistance marker control strain DC88 (data not shown).

[0310] The genR antibiotic marker gene was then removed from the pDOW 1264-2 (**FIG. 3**) to create an antibiotic-marker-free plasmid with proC and lacI. Removing the genR gene was accomplished by restriction digestion of pDOW1264-2 with BamHI, purification of the 6.1 kB fragment, ligation to itself, and electroporation into the proC(−) *P. fluorescens* host PFG1016. Isolates were checked by restriction digestion using EcoRI. The resulting plasmid was named pDOW1306-6. Analytical restriction digests with EcoRI and sequencing across the BamHI junction verified the identity of the plasmid and the proper orientations of the genes therein.

[0311] Sequencing was performed by The Dow Chemical Company. The proC sequence is presented within SEQ ID NO:4.

Construction of Target Enzyme Expression Plasmid Containing a pyrF Marker in Place of an Antibiotic Resistance Marker

[0312] The antibiotic-marker-free production plasmid, pDOW1269-2, containing a target enzyme-encoding gene under control of a tac promoter, was constructed by restriction digestion of pDOW1249-2 with PvuI to remove the tetR/tetA genes. Derived from pMYC5088 by insertion of the pyrF gene from MB214, pDOW1249-2 was prepared as described in Example 1. The 10.6 kB PvuI fragment was gel-purified, ligated to itself, transformed into PFG118/pCN51lacI by electroporation and spread on M9 glucose medium containing kanamycin (to retain the pCN51lacI). Plasmid DNA was isolated and analytical restriction digests with NcoI were carried out; two isolates showed a restriction digest that was consistent with the expected bands. Both isolates were sequenced across the PvuI junction, which verified the identity of the plasmids and the proper orientations of the genes therein.

Construction of a Pseudomonas fluorescens Strain with Genomic Deletions of pyrF and proC

[0313] PFG118, a *P. fluorescens* MB 101 strain with a deletion of pyrF, was described in Example 1.

Construction of pDOW 1261-2, a Vector for Gene Replacement and Deletion

[0314] The vector pDOW1261-2 was designed to create clean deletions of genomic DNA, using marker exchange by the cross-in/cross-out method (Toder 1994; Davison 2002), by combining the following properties:

[0315] a ColEI replication origin that functions only in *E.coli* and not in *P. fluorescens;*

[0316] a selectable marker (tetR/tetA) for integration of the plasmid into the chromosome;

[0317] a counterselectable marker (pyrF) that allows for selection for loss of the inserted plasmid (as long as the host strain is pyrF-); cells that lose the pyrF gene are resistant to 5-FOA; and

[0318] a blunt-end cloning site, SrfI, which has an uncommon 8 bp recognition site - if the desired insert lacks the site, the efficiency of insertion can be increased by adding SrfI (Stratagene, La Jolla Calif.) to the ligation reaction to re-cleave vectors that ligate without an insert.

[0319] To construct this vector, a 5 kB PstI to EcoRI fragment containing the tetR, tetA, and pyrF genes was cloned into pCRScriptCAM (Stratagene, La Jolla Calif.) that had been digested with PstI and EcoRI, creating pDOW 1261-2.

Construction of a Vector to Delete proC from the Chromosome

[0320] To construct a deletion of proC, the copies of the flanking regions upstream and downstream of the proC gene were joined together by PCR, and then cloned into the pDOW1261-2 gene replacement vector. The proC7 primer, which bridges the proC ORF, was designed to delete the entire coding sequence from the ATG start codon to the TAG stop codon. An additional 16 bp downstream of the stop codon was also included in the deletion.

[0321] To make the PCR fusion of regions upstream and downstream from proC, the Megaprimer method of PCR amplification was used (Barik 1997). To make the megaprimer, the 0.5 kB region directly upstream of the proC open reading frame was amplified by PCR from MB214 genomic DNA, using primers proC5 and proC7. Primer proC7 overlaps the regions upstream and downstream of the proC ORF. The polymerase chain reaction was carried out with 1 uM of primers, 200 uM each of the four dNTPs, and Herculase high-fidelity polymerase (Stratagene, La Jolla Calif.) in the buffer recommended by the vendor. Herculase is a high-fidelity enzyme that consists mostly of Pfu polymerase, which leaves blunt ends. The amplification program was 95° C. for 2 min, 30 cycles of 95° C. for 30 sec, 50° C. for 30 sec, and 72° C. for 1 min per kB, followed by 10 min at 72° C. The amplified products were separated by 1% agarose gel electrophoresis in TBE and visualized using ethidium bromide. A gel slice containing the DNA was cut from the gel and purified as above The 1.3 kB region downstream from the proC gene was amplified using primers proC3 and proC6, to serve as a template for subsequent reactions. The same amplification protocol was used, except for an annealing temperature of 60° C. The reaction was checked on an agarose gel, and then purified using the StrataPrep PCR Purification Kit (Stratagene, La Jolla Calif.).

[0322] In the second step to make the deletion fusion, the megaprimer was used as one of the primers in a PCR reaction along with primer proC6, and with the proC3-proC6 PCR reaction as the template. An annealing temperature of 61° C. and extension time of 2 min was used. The 1 kB PCR product was purified and blunt-end ligated into the suicide vector pDOW1261-2 that had been digested with SrfI. SrfI was included in the ligation in order to decrease background caused by re-ligation of the vector, as according to instructions from the manufacturer (pCRScriptCam Cloning Kit—Stratagene, La Jolla Calif.). The ligation was transformed into DH10 β (Gibco BRL Life Technologies, now Invitrogen, Carlsbad Calif.) by electroporation (2 mM gap cuvette, 25 μF, 2.25 kV, 200 Ohms) (Artiguenave et al. 1997), and isolates were screened using the DraIII restriction enzyme. The PCR amplified region of each isolate was sequenced by The Dow Chemical Company; isolate pDOW1305-6 was verified as containing the correct genomic DNA sequence.

[0323] Formation of the *P. fluorescens* pvrF-proC Double Deletion

[0324] To make a doubly deleted strain, PFG118 was transformed with pDOW1305-6 by electroporation as described above. Analytical PCR on the colonies with primers proC8 and the M13/pUC Reverse Sequencing Primer (−48) (which hybridizes to the plasmid only) (New England Biolabs, Beverly Mass.), using HotStarTaq (Qiagen, Valencia Calif.), an annealing temperature of 59° C. and an extension time of 4 min, showed that 9 out of 22 isolates had the plasmid integrated into the region upstream from proC, and 7 out of 22 had the plasmid integrated downstream (data not shown). Three of each orientation were purified to single colonies. The three isolates PFG118::1305-6.1, -6.8, -6.10 have an insertion in the region upstream, and the three isolates PFG118::1305-6.2, -6.3, -6.9 have an insertion in the region downstream.

[0325] To select for cells that have carried out a homologous recombination between the plasmid and the chromosome genes thereby leaving a deletion, PFG118::1305-6.1 and -6.2 were grown to stationary phase in 50 mL of LB with uracil and proline supplementation and then plated on LB-5-FOA with uracil and proline supplementation. Cells that lose the integrated plasmid by recombination also lose the pyrF gene, and are therefore expected to be resistant to 5-FOA which would otherwise be converted into a toxic compound. PCR analysis with proC8 and proC9 was carried out to distinguish between cells that had lost the plasmid and regenerated the original sequence, and those that had left the deletion. Two isolates with the expected 1.3 kB band were chosen from each of the two selections and named PFG1013, PFG1014, PFG1015 and PFG1016 (also known as DC164). All four isolates were unable to grow on M9 glucose unless both proline and uracil were added, and were tetracycline-sensitive. The genomic region of PFG118 (wild type proC) and PFG1016 (proC deletion) was amplified by PCR (primers proC8 and proC9, HotStarTaq polymerase, 63° C. annealing and 3 min extension) and sequenced. The region between proC5 and proC6 of strain PFG1016 was identical to the parent, except for the expected 835 bp deletion.

Construction of a Dual Auxotrophic Selection Marker Expression System PFG 1016/pDOW 1306-6 pDOW 1269-2

[0326] Plasmids were isolated from strain PFG118 pCN51lacI pDOW1269-2 by HISPEED Plasmid Midi Kit (Qiagen, Valencia Calif.). The pDOW1269-2 was partially purified from the pCN51lacI by agarose gel electrophoresis and then electroporated into PFG1016 pDOW1306-6 . Transformants were selected on M9/glucose without supplementation. Because there was a possibility that some of the pCN51lacI contaminating the pDOW1269-2 preparation would also be cotransformed into the cells, three isolates from each transformation were tested for sensitivity to kanamycin, the antibiotic marker carried on pCN51lac; all six were found sensitive. All six strains were found to express the target enzyme, in a test of target enzyme activity. PCR analysis showed that all six also contained the chromosomal proC deletion.

[0327] Restriction digestion of plasmids isolated from the transformants was consistent with the expected pattern.

Performance Testing of the Dual Auxotrophic Marker Expression System in Shake Flasks

[0328] The six strains were then tested in shake flasks as described above in Example 1. Induction of target enzyme expression was initiated at 26 hours by addition of IPTG. The OD_{575} for all six strains was comparable to that of the dual-antibiotic-resistance marker expression system control, DC88. Target enzyme production levels in all six were also comparable to that of the control, as assessed by SDS-PAGE. The two strains that achieved the highest OD_{575}, strains 1046 and 1048, were selected for further characterization.

Performance Testing of the Dual Auxotrophic Marker Expression System in 20-L Bioreactors

[0329] Strains 1046 and 1048 were subsequently tested in 20-L bioreactors. Induction of target enzyme expression was initiated at 26 hours by addition of IPTG. Both strains achieved performance levels within the normal range for the DC88 control strain, for both OD_{575} and target enzyme activity. The performance averages of these two strains are shown in **FIG. 1**. Restriction digests of plasmids prepared from samples taken at the seed stage and at a time just before the 26-hour start of induction showed a pattern consistent with that expected. Analytical PCR of genomic DNA carried out on the same samples demonstrated the retention of the proC deletion and the pyrF deletion. Aliquots of the 25 hr samples were plated on tetracycline-, gentamycin-, or kanamycin-supplemented media; no cell growth was observed, thus confirming the absence of antibiotic resistance gene activity.

[0330] Analysis of strain 1046 (also known as DC167) in 20-L bioreactors was repeated twice with similar results. Plasmid stability at the seed stage and after 25 hours of fermentation (immediately before induction) was tested by replica plating from samples that had been diluted and plated on complete media. Both plasmids were present in more than 97% of the colonies examined, indicating the lack of cross feeding revertants able to survive without the plasmid and the stable maintenance of the expression vector in *Pseudomonas fluorescens*.

Results

[0331] Both of the pyrF expression systems performed as well as the control system in which only antibiotic resistance markers were used (**FIG. 1**). For the control strain, there is no negative effect of cross-feeding, since any importation of exogenous metabolites from lysed cells does not decrease or remove the selection pressures provided by the antibiotics in the medium. The expected decreases in performance expected as a result of cross-feeding on the two pyrF expression systems were surprisingly not observed.

Example 3

Chromosomal Integration of lacI. $lacI^Q$ and $lacI^{Q1}$ in *P. fluorescens*

[0332] Three *P. fluorescens* strains have been constructed, each with one of three different *Escherichia coli* lacI alleles, lacI (SEQ ID NO:9), $lacI^Q$ (SEQ ID NO: 11), and $lacI^{Q1}$ (SEQ ID NO:12), integrated into the chromosome. The three strains exhibit differing amounts of LacI repressor accumulation. Each strain carries a single copy of its lacI gene at the levansucrase locus (SEQ ID NO:13) of *P. fluorescens* DC36, which is an MB101 derivative (see TD Landry et al., "Safety evaluation of an α-amylase enzyme preparation derived from the archaeal order Thermococcales as expressed in *Pseudomonas fluorescens* biovar I,"*Regulatory Toxicology and Pharmacology* 37(1): 149-168(2003)) formed by deleting the pyrF gene thereof, as described above.

[0333] No vector or other foreign DNA sequences remain in the strains. The strains are antibiotic-resistance-gene free and also contain a pyrF deletion, permitting maintenance, during growth in uracil un-supplemented media, of an expression plasmid carrying a pyrF+ gene. Protein production is completely free of antibiotic resistance genes and antibiotics.

[0334] MB214 contains the lacI-lacZYA chromosomal insert described in U.S. Pat. No. 5,169,760. MB214 also

contains a duplication in the C-terminus of the LacI protein, adding about 10 kDa to the molecular weight of the LacI repressor.

Construction of Vector pDOW1266-1 for Insertion of Genes into the Levansucrase Locus

[0335] Plasmid pDOW1266-1 was constructed by PCR amplification of the region upstream of and within the *P. fluorescens* gene for levansucrase (SEQ ID NO:13), replacing the start codon with an XbaI site, using the Megaprimer method, see A Barik, "Mutagenesis and Gene Fusion by Megaprimer PCR," in BA White, *PCR Cloning Protocols* 173-182 (1997) (Humana). PCR was performed using HERCULASE polymerase (Stratagene, Madison Wis., USA) using primers LEV1 (SEQ ID NO:34) and LEV2 (SEQ ID NO:35), and *P. fluorescens* MB214 genomic DNA as a template (see below for oligonucleotide sequences). Primer LEV2 (SEQ ID NO:35) contains the sequence that inserts an XbaI site. The reaction was carried out at 95° C. for 2 min, 35 cycles of [95° C. for 30 sec, 58° C. for 30 sec, 72° C. for 1 min], followed by 10 min at 72° C. The 1 kB product was gel purified and used as one of the primers in the next reaction, along with LEV3 (SEQ ID NO:36), using MB214 genomic DNA as a template and the same conditions except that extension time was 2 min. The 2 kB product was gel purified and re-amplified with LEV2 (SEQ ID NO: 35) and LEV3 (SEQ ID NO. 36) in order to increase the quantity.

[0336] Oligonucleotides Used

LEV1
5'-TTCGAAGGGGTGCTTTTTCTAGAAGTAAGTC (SEQ ID NO: 34)
TCGTCCATGA

LEV2
5'-CGCAAGGTCAGGTACAACAC (SEQ ID NO: 35)

LEV3
5'-TACCAGACCAGAGCCGTTCA (SEQ ID NO: 36)

LEV7
5'-CTACCCAGAACGAAGATCAG (SEQ ID NO: 37)

LEV8
5'-GACTCAACTCAATGGTGCAGG (SEQ ID NO: 38)

BglXbaLacF
5'-AGATCTCTAGAGAAGGCGAAGCGGCATGCAT (SEQ ID NO: 39)
TTACG

lacIR4
5'-ATATTCTAGAGACAACTCGCGCTAACTTACA (SEQ ID NO: 40)
TTAATTGC

Lacpro9
5'-ATATTCTAGAATGGTGCAAAACCTTTCGCGG (SEQ ID NO: 41)
TATGGCATGA

LacIQF
5'-GCTCTAGAAGCGGCATGCATTTACGTTGACA (SEQ ID NO: 42)
CC

LacINXR
5'-AGCTAGCTCTAGAAAGTTGGGTAACGCCAGG (SEQ ID NO: 43)
GT

lacIQ1
5'-AGTAAGCGGCCGCAGCGGCATGCATTTACGT (SEQ ID NO: 44)
TGACACCACCTTTCGCGGTATGGCATG

-continued

The Oligos Below were Used
for Analytical Sequencing Only
lacIF1
5'-ACAATCTTCTCGCGCAACGC (SEQ ID NO: 45)

lacIF2
5'-ATGTTATATCCCGCCGTTAA (SEQ ID NO: 46)

lacIR1
5'-CCGCTATCGGCTGAATTTGA (SEQ ID NO: 47)

lacIR2
5'-TGTAATTCAGCTCCGCCATC (SEQ ID NO: 48)

SeqLev5AS
5'-TATCGAGATGCTGCAGCCTC (SEQ ID NO: 49)

SeqLev3S
5'-ACACCTTCACCTACGCCGAC (SEQ ID NO: 50)

LEV10
5'-TCTACTTCGCCTTGCTCGTT (SEQ ID NO: 51)

[0337] The LEV2 - LEV3 amplification product was cloned into the SrfI site of pDOW1261-2, a suicide vector that contains *P. fluorescens* pyrF+ gene as a selection marker to facilitate selection for cross-outs. The new plasmid was named pDOW1266-1. The amplified region was sequenced.

Cloning the lacI Genes into Insertion Vector pDOW1266-1

[0338] The *E.coli* lacI gene was amplified from pCN51lacI with primers BglXbaLacF (SEQ ID 10 NO:39) and lacIR4 (SEQ ID NO. 40), using HERCULASE polymerase (annealing at 62° C. and extension time of 2 min). After gel purification and digestion with XbaI, the lacI gene was cloned into the XbaI site of pDOW1266-1, to make pDOW1310. The $lacI^{Q}$ gene was created by PCR amplification using pCN51lacI as a template with 15 primers lacpro9 (SEQ ID NO. 41) and lacIR4 (SEQ ID NO. 40), using HERCULASE polymerase (annealing at 62° C. and extension time of 2 min). After gel purification and digestion with XbaI, it was cloned into the XbaI site of pDOW1266-1, to make pDOW1311.

[0339] The $lacI^{Q1}$ gene was created by amplifying the lacI gene from *E.coli* K12 (ATCC47076) using primers lacIQ1 (SEQ ID NO. 44) and lacINXR (SEQ ID NO. 43) and cloned into pCR2.1 Topo (Invitrogen, Carlsbad, Calif., USA), to make pCR2-lacIQ1. The $lacI^{Q1}$ gene was reamplified from pCR2-lacIQ1 using primers lacIQF (SEQ ID NO. 42) and lacINXR (SEQ ID NO. 43) with Herculase polymerase (61° C. annealing, 3 min extension time, 35 cycles). After gel purification and digestion with XbaI, the PCR product was cloned into the XbaI site of pDOW1266-1, to make pDOW1309.

[0340] The PCR amplified inserts in pCR2-lacIQ1, pDOW1310, pDOW1311, and pDOW1309 were sequenced (using primers lacIF1 (SEQ ID NO:45), lacIF2 (SEQ ID NO. 46), lacIR1 (SEQ ID NO. 47), lacIR2 (SEQ ID NO. 48), SeqLev5AS (SEQ ID NO. 49), SeqLev3S (SEQ ID NO. 50), and LEV10 (SEQ ID NO. 51)) to insure that no mutations had been introduced by the PCR reaction. In each case, an orientation was chosen in which the lacI was transcribed in the same direction as the levansucrase gene. Although the levansucrase promoter is potentially able to control tran-

scription of lacI, the promoter would only be active in the presence of sucrose, which is absent in the fermentation conditions used.

Construction of *P. fluorescens* Strains with Integrated lacI Genes at the Levansucrase Locus

[0341] The vectors pDOW1309, pDOW1310, and pDOW1311 were introduced into DC36 by electroporation, first selecting for integration of the vector into the genome with tetracycline resistance. Colonies were screened to determine that the vector had integrated at the levansucrase locus by PCR with primers LEV7 (SEQ ID NO. 37) and M13R (from New England Biolabs, Gloucester Mass., USA). To select for the second cross-over which would leave the lacI gene in the genome, the isolates were grown in the presence of 5'-fluoroorotic acid and in the absence of tetracycline. Recombination between the duplicated regions in the genome either restores the parental genotype, or leaves the lacI gene. The resulting isolates were screened for sensitivity to tetracycline, growth in the absence of uracil, and by PCR with primers LEV7 (SEQ ID NO. 37) and LEV8 (SEQ ID NO. 38). The names of the new strains are shown in Table 17. To obtain sequence information for genomic regions, PCR products were sequenced directly, see E Werle, "Direct sequencing of polymerase chain reaction products, "*Laboratory Methods for the Detection of Mutations and Polymorphisms in DNA* 163-174 (1997). For each strain, the sequencing confirmed the identity of the promoter, the orientation of the lacI variant relative to the flanking regions, and whether there were any point mutations relative to the parental sequence. The sequences of DC202 and DC206 were as expected. The sequence of DC204 showed a point mutation within the levansucrase open reading frame, downstream of $lacI^Q$, which did not change any coding sequence and therefore is inconsequential.

TABLE 17

P. FLUORESCENS STRAINS WITH LACI ALLELES INTEGRATED INTO THE GENOME

Strain Designation	Plasmid used to make the lacI insertion	Genotype
DC202	pDOW1310-1	pyrF lev::lacI
DC204	pDOW1311-4	pyrF lev::$lacI^Q$
DC206	pDOW1309oriA	pyrF lev::$lacI^{Q1}$

Analysis of Relative Concentration of LacI in the lacI Integrants, Compared to pCN51lacI

[0342] UnBlot is a method analogous to Western analysis, in which proteins are detected in the gel without the need for transfer to a filter. The technique was carried out following the directions from Pierce Biotechnology (Rockford, Ill., USA), the manufacturer. Analysis using UnBlot showed that the amount of LacI in each of the new integrant strains was higher than in MB214. MB214 contains the lacI-lacZYA insert described in U.S. Pat. 5,169,760. The relative concentration of LacI in the $lacI^Q$ and $lacI^{Q1}$ integrants was about the same as in strains carrying pCN51lacI, the multicopy plasmid containing lacI. See **FIG. 5**.

[0343] A dilution series was carried out in order to assess more precisely the relative difference in LacI concentration in MB214, DC202 (lacI integrated) and DC206 ($lacI^{Q1}$ integrated). MB101pCN51lacI, DC204 and DC206 have about 100 times more LacI than MB214, whereas DC202 has about 5 times more.

Example 4

Nitrilase Expression and Transcription

[0344] Strain DC140 was constructed by introducing into *P. fluorescens* MB214 a tetracycline- resistant broad-host-range plasmid, pMYC1803 (WO 2003/068926), into which a nitrilase gene (G DeSanthis et al., *J Amer. Chem. Soc.* 125:11476-77 (2003)), under the control of the Ptac promoter, had been inserted. In order to compare regulation of un-induced expression of the target gene in DC202 and DC206 with MB214, the same nitrilase gene was cloned onto a pMYC1803 derivative where the tetracycline-resistance gene has been replaced by a pyrF selection marker. The new plasmid, pDOW2415, was then electroporated into DC202 and DC204, resulting in DC239 and DC240, respectively. DC140, DC239 and DC240 were cultured in 20 L fermentors by growth in a mineral salts medium fed with glucose or glycerol, ultimately to cell densities providing biomasses within the range of about 20 g/L to more than 70 g/L dry cell weight (See WO 2003/068926). The gratuitous inducer of the Ptac promoter, IPTG, was added to induce expression.

[0345] The ratio of pre-induction nitrilase activities of DC140 to DC239 to DC240 was 6:2:1. RNA analysis by Northern blots of the same samples revealed the same ranking of derepression. Based on densitometric measurements, the ratio of un-induced transcript levels of DC140:DC239:DC240 was 2.4:1.4:1.0. Shortly after induction (30 min) with 0.3mM IPTG, the levels of transcript of all the strains were the same. Post-induction nitrilase productivity rates were also comparable. This indicated that the concentration of inducer used was sufficient to fully induce the Ptac promoter in these three strains despite their different LacI protein levels. However, fermentations of the most derepressed strain, DC140, suffered significant cell lysis accompanied with loss of nitrilase activity after approximately 24 hours post-induction. Induction of the improved, more tightly regulated strains, DC239 and DC240, could be extended to more than 48 hours post induction, while maintaining high nitrilase productivity, with the ultimate result of a doubling of nitrilase yields. See **FIG. 6**.

Results

[0346] The examples indicate It that the use of a LacI-encoding gene other than as part of a whole or truncated Plac-lacI-lacZYA operon in Pseudomonads resulted in substantially improved repression of pre-induction recombinant protein expression, higher cell densities in commercial-scale fermentation, and higher yields of the desired product in comparison with previously taught lacI-lacZYA Pseudomonad chromosomal insertion (U.S. Pat. No. 5,169,760). The results also indicated that the lacI insertion is as effective in producing LacI repressor protein in *Pseudomonas fluorescens* , thereby eliminating the need to maintain a separate plasmid encoding a LacI repressor protein in the cell and reducing potential production inefficiencies caused by such maintenance.

[0347] In addition to being antibiotic free, derepression during the growth stage in DC239 and DC240 was up to 10 fold less than the MB214 strain. Pre-induction nitrilase activity levels of DC239 and DC240 averaged 0.4 U/ml in more than 13 separate fermentations, and cell density and nitrilase expression in DC239 and DC240 did not decay during extended induction phase, as it did in DC140. Given the higher derepression, DC239 and DC240 fermentation

runs decreased the time of the growth phase by more than 30%, reducing fermentation costs.

Example 5

Construction of tac Promoter with a Single Optimal lac Operator and with Two lac Operators

[0348] The native tac promoter only has a single native lac operator, AATTGTGAGCGGATAACAATT, at the O1 position (**FIG. 4**). In the first construct, pDOW1418, the native operator was replaced by the more symmetrical lacOid operator sequence 5'-AATTGTGAGC GCTCACAATT - 3' (SEQ. ID. NO. 14) (J R. Sadler, H. Sasmor and J L. Betz. PNAS. 1983 Nov.; 80 (22): 6785-9). A 289 bp HindIII/ SpeI fragment containing the tac promoter and the native lacO sequence was removed from a pMYC1803 derivative, pDOW2118, and replaced by a HindIII/SpeI fragment isolated from an SOE PCR amplification product containing the symmetrical lacOid sequence. The SOE PCR primers (RC-3 and RC-9) incorporated 4 nucleotide changes that produced the optimized/symmetrical lacO sequence (three base pair substitutions and one base pair deletion). The HindIII/SpeI promoter fragment of the resulting plasmid, pDOW2201, was cloned into the nitrilase expression plasmid based on pMYC1803, to replace the native tac promoter, resulting in pDOW1414. This expression cassette was then transferred onto the pyrF(+) plasmid pDOW1269, resulting in pDOW1418 by exchanging DraI/XhoI fragments. Plasmid pDOW1418 was then transformed into host strain DC206, resulting in strain DC281 (See **FIG. 4**).

[0349] Oligonucleotides Used

```
RC-3
5'-GTGAGCGCTCACAATTCCACACAGGAAA   (SEQ ID NO: 52)
ACAG

RC-4
5'-TTCGGGTGGAAGTCCAGGTAGTTGGCGG   (SEQ ID NO: 53)
TGTA

RC-9
5'-GAATTGTGAGCGCTCACAATTCCACACA   (SEQ ID NO: 54)
TTATACGAGC

RC-10
5'-ATTCAGCGCATGTTCAACGG           (SEQ ID NO: 55)
```

[0350] In the second construct, pDOW1416, the lacOid operator, 5'-AATTGTGAGC GCTCACAATT-3' (SEQ ID. No. 14), was inserted 52 nucleotides up-stream (5') of the existing native lacO1 by PCR. PCR amplification of the promoter region using the Megaprimer method was performed using a pMYC1803 derivative, pMYC5088, and the following primers AKB-1 and AKB-2 as a first step. The resulting PCR product was combined with primer AKB-3 in a second round of PCR amplification using the same template. After purification and digestion with HindIII and SpeI, the promoter fragment containing the dual operators was cloned into the HindIII and SpeI sites of plasmid pMYC5088 resulting in pDOW1411. Introduction of the second operator introduced a unique MfeI site immediately upstream of the optimal operator. The XhoI/SpeI vector fragment with promoter regions of pDOW1411 was then ligated with the compatible fragment of the pMYC1803 derivative bearing the nitrilase gene, forming pDOW1413. Subsequent ligation of the MfeI/XhoI expression cassette fragment of pDOW1413 to the compatible vector fragment of pDOW1269 resulted in pDOW1416; which when transformed into DC206, formed the strain DC262.

[0351] Oligonucleotides Used

```
AKB-1
5'-ACGGTTCTGGCAAACAATTGTGAGCGCTCAC (SEQ ID NO: 56)
AATTTATTCTGAAATGAGC

AKB-2
5'-GCGTGGGCGGTGTTTATCATGTTC        (SEQ ID NO: 57)

AKB-3
5'-TACTGCACGCACAAGCCTGAACA         (SEQ ID NO: 58)
```

Nitrilase Derepression

[0352] Northern blot analysis was performed pre and post induction on MB214, DC202, and DC206. MB214, DC202, and DC206 were transformed with a nitrilase expression vector containing the wild type lacO sequence in the O_1 position 3' of the tac promoter, creating MB214 wtO_1, DC202wtO_1 (DC239), and DC206wtO_1 (DC240), as described above. DC206 was transformed with a nitrilase expression vector containing a lacOid sequence in place of the wild type lacO sequence at the O_1 position 3' of the tac promoter as described above, creating DC206Oid (DC281). DC206 was also transformed with a nitrilase expression vector containing a wild type lacO sequence at the O_1 position 3' of the tac promoter and a lacOid sequence at the O_3 position 5' of the tac promoter, creating the dual lacO containing DC206wtO_1 O_3id (DC263).

[0353] Northern blot analysis indicated a greater repression by the strain containing the Dual lacO sequence (DC206wtO_1 O_3id (DC263)) cassette prior to induction. The greater repression of pre-induction expression is especially useful when producing toxic proteins, since basal levels of pre-induction toxic proteins result in the delayed entry of the cell into the growth phase, and, potentially, lower overall yields of the product.

SEQUENCE LISTING

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ccaagtgtcg aaacggatat tggcccgctg ttcggtgcgg tcatcacgca cccggcgcag 300

gatggtctgg atacgcgcga ccagttcccg gggttcgaac ggcttggaca tatagtcgtc 360

tgcccccagt tccaggccga tgatgcggtc ggtgggttcg cagcgggcgg tgagcatcag 420

gatcggaatg tccgattcgg cgcgcagcca gcggcacaat gtcagcccgt cttcgcccgg 480

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ttcacgtagt cccccaccgc gaagttcgga tggttcgagg caagcacctt gcctacgccc 2580

agggcgcgca tcacttcgcc gatgcctacc ggtgggatgt aggacttgcc ttcattcatc 2640

cagccacgca 2650

<210> SEQ ID NO 2
<211> LENGTH: 232
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 2

Met Ser Val Cys Gln Thr Pro Ile Ile Val Ala Leu Asp Tyr Pro Thr
1 5 10 15

Arg Asp Ala Ala Leu Lys Leu Ala Asp Gln Leu Asp Pro Lys Leu Cys
20 25 30

Arg Val Lys Val Gly Lys Glu Leu Phe Thr Ser Cys Ala Ala Glu Ile
35 40 45

Val Gly Thr Leu Arg Asp Lys Gly Phe Glu Val Phe Leu Asp Leu Lys
50 55 60

Phe His Asp Ile Pro Asn Thr Thr Ala Met Ala Val Lys Ala Ala Ala
65 70 75 80

Glu Met Gly Val Trp Met Val Asn Val His Cys Ser Gly Gly Leu Arg
85 90 95

Met Met Ser Ala Cys Arg Glu Val Leu Glu Gln Arg Ser Gly Pro Lys
100 105 110

Pro Leu Leu Ile Gly Val Thr Val Leu Thr Ser Met Glu Arg Glu Asp
115 120 125

Leu Ala Gly Ile Gly Leu Asp Ile Glu Pro Gln Val Gln Val Leu Arg
130 135 140

Leu Ala Ala Leu Ala Gln Lys Ala Gly Leu Asp Gly Leu Val Cys Ser
145 150 155 160

Ala Leu Glu Ala Gln Ala Leu Lys Asn Ala His Pro Ser Leu Gln Leu
165 170 175

Val Thr Pro Gly Ile Arg Pro Thr Gly Ser Ala Gln Asp Asp Gln Arg
180 185 190

Arg Ile Leu Thr Pro Arg Gln Ala Leu Asp Ala Gly Ser Asp Tyr Leu
195 200 205

Val Ile Gly Arg Pro Ile Ser Gln Ala Ala Asp Pro Ala Lys Ala Leu
210 215 220

Ala Ala Val Val Ala Glu Ile Ala
225 230

<210> SEQ ID NO 3
<211> LENGTH: 696
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas fluorescens

-continued

<400> SEQUENCE: 3

atgtccgtct gccagactcc tatcatcgtc gccctggatt accccacccg tgacgccgca 60

ctgaagctgg ctgaccagtt ggaccccaag ctttgccggg tcaaggtcgg caaggaattg 120

ttcaccagtt gcgcggcgga aatcgtcggc accctgcggg acaaaggctt cgaagtgttc 180

ctcgacctca aattccatga catccccaac accacggcga tggccgtcaa agccgcggcc 240

gagatgggcg tgtggatggt caatgtgcac tgctccggtg gcctgcgcat gatgagcgcc 300

tgccgcgaag tgctggaaca gcgcagcggc cccaaaccgt tgttgatcgg cgtgaccgtg 360

ctcaccagca tggagcgcga agacctggcg ggcattggcc tggatatcga gccgcaggtg 420

caagtgttgc gcctggcagc cctggcgcag aaagccggcc tcgacggcct ggtgtgctca 480

gccctggaag cccaggccct gaaaaacgca catccgtcgc tgcaactggt gacaccgggt 540

atccgtccta ccggcagcgc ccaggatgac cagcgccgta tcctgacccc gcgccaggcc 600

ctggatgcgg gctctgacta cctggtgatc ggccggccga tcagccaggc ggcggatcct 660

gcaaaagcgt tggcagcggt cgtcgccgag atcgcc 696

<210> SEQ ID NO 4
<211> LENGTH: 834
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 4

atgaagcaat atctcgaact actgaacgac gtcgtgacca atggattgac caagggcgat 60

cgcaccggca ccggcaccaa agccgtattt gcccgtcagt atcggcataa cttggccgac 120

ggcttcccgc tgctgaccac caagaagctt catttcaaaa gtatcgccaa cgagttgatc 180

tggatgttga gcggcaacac caacatcaag tggctcaacg aaaatggcgt gaaaatctgg 240

gacgagtggg ccaccgaaga cggcgacctg ggcccggtgt acggcgagca atggaccgcc 300

tggccgacca aggacggcgg caagatcaac cagatcgact acatggtcca caccctcaaa 360

accaacccca acagccgccg catcctgttt catggctgga acgtcgagta cctgccggac 420

gaaaccaaga gcccgcagga gaacgcgcgc aacggcaagc aagccttgcc gccgtgccat 480

ctgttgtacc aggcgttcgt gcatgacggg catctgtcga tgcagttgta tatccgcagc 540

tccgacgtct tcctcggcct gccgtacaac accgccgcgt tggccttgct gactcacatg 600

ctggctcagc aatgcgacct gatccctcac gagatcatcg tcaccaccgg cgacacccat 660

gcttacagca accacatgga acagatccgc acccagctgg cgcgtacgcc gaaaaagctg 720

ccggaactgg tgatcaagcg taaacctgcg tcgatctacg attacaagtt tgaagacttt 780

gaaatcgttg gctacgacgc cgacccgagc atcaaggctg acgtggctat ctga 834

<210> SEQ ID NO 5
<211> LENGTH: 277
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 5

Met Lys Gln Tyr Leu Glu Leu Leu Asn Asp Val Val Thr Asn Gly Leu
1 5 10 15

Thr Lys Gly Asp Arg Thr Gly Thr Gly Thr Lys Ala Val Phe Ala Arg
20 25 30

-continued

```
Gln Tyr Arg His Asn Leu Ala Asp Gly Phe Pro Leu Leu Thr Thr Lys
        35                  40                  45

Lys Leu His Phe Lys Ser Ile Ala Asn Glu Leu Ile Trp Met Leu Ser
    50                  55                  60

Gly Asn Thr Asn Ile Lys Trp Leu Asn Glu Asn Gly Val Lys Ile Trp
65                  70                  75                  80

Asp Glu Trp Ala Thr Glu Asp Gly Asp Leu Gly Pro Val Tyr Gly Glu
                85                  90                  95

Gln Trp Thr Ala Trp Pro Thr Lys Asp Gly Gly Lys Ile Asn Gln Ile
            100                 105                 110

Asp Tyr Met Val His Thr Leu Lys Thr Asn Pro Asn Ser Arg Arg Ile
        115                 120                 125

Leu Phe His Gly Trp Asn Val Glu Tyr Leu Pro Asp Glu Thr Lys Ser
    130                 135                 140

Pro Gln Glu Asn Ala Arg Asn Gly Lys Gln Ala Leu Pro Pro Cys His
145                 150                 155                 160

Leu Leu Tyr Gln Ala Phe Val His Asp Gly His Leu Ser Met Gln Leu
                165                 170                 175

Tyr Ile Arg Ser Ser Asp Val Phe Leu Gly Leu Pro Tyr Asn Thr Ala
            180                 185                 190

Ala Leu Ala Leu Leu Thr His Met Leu Ala Gln Gln Cys Asp Leu Ile
        195                 200                 205

Pro His Glu Ile Ile Val Thr Thr Gly Asp Thr His Ala Tyr Ser Asn
    210                 215                 220

His Met Glu Gln Ile Arg Thr Gln Leu Ala Arg Thr Pro Lys Lys Leu
225                 230                 235                 240

Pro Glu Leu Val Ile Lys Arg Lys Pro Ala Ser Ile Tyr Asp Tyr Lys
                245                 250                 255

Phe Glu Asp Phe Glu Ile Val Gly Tyr Asp Ala Asp Pro Ser Ile Lys
            260                 265                 270

Ala Asp Val Ala Ile
        275
```

<210> SEQ ID NO 6
<211> LENGTH: 1853
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 6

```
gcccttgagt tggcacttca tcggccccat tcaatcgaac aagactcgtg ccatcgccga     60

gcacttcgct tgggtgcact ccgtggaccg cctgaaaatc gcacaacgcc tgtccgaaca    120

acgcccggcc gacctgccgc cgctcaatat ctgcatccag gtcaatgtca gtggcgaagc    180

cagcaagtcc ggctgcacgc ccgctgacct gccggccctg gccacagcga tcagcgccct    240

gccgcgcttg aagctgcggg gcttgatggc gattcccgag ccgacgcaag accgggcgga    300

gcaggatgcg gcgttcgcca cggtgcgcga cttgcaagcc agcttgaacc tggcgctgga    360

cacactttcc atgggcatga gccacgacct tgagtcggcc attgcccaag gcgccacctg    420

ggtgcggatc ggtaccgccc tgtttggcgc ccgcgactac ggccagccgt gaaatggctg    480

acatccctcg aaataaggac ctgtcatgag caacacgcgt attgccttta tcggcgccgg    540

taacatggcg gccagcctga tcggtggcct gcgggccaag ggcctggacg ccgagcagat    600
```

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```
ccgcgccagc gaccccggtg ccgaaacccg cgagcgcgtc agagccgaac acggtatcca    660

gaccttcgcc gataacgccg aggccatcca cggcgtcgat gtgatcgtgc tggcggtcaa    720

gccccaggcc atgaaggccg tgtgcgagag cctgagcccg agcctgcaac cccatcaact    780

ggtggtgtcg attgccgctg gcatcacctg cgccagcatg accaactggc tcggtgccca    840

gcccattgtg cgctgcatgc ccaacacccc ggcgctgctg cgccagggcg tcagcggttt    900

gtatgccact ggcgaagtca ccgcgcagca acgtgaccag gcccaggaac tgctgtctgc    960

ggtgggcatc gccgtgtggc tggagcagga acagcaactg gatgcggtca ccgccgtctc   1020

cggcagcggc ccggcttact tcttcctgtt gatcgaggcc atgacggccg caggcgtcaa   1080

gctgggcctg ccccacgacg tggccgagca actggcggaa caaaccgccc tgggcgccgc   1140

caagatggcg gtcggcagcg aggtggatgc cgccgaactg cgccgtcgcg tcacctcgcc   1200

aggtggtacc acacaagcgg ctattgagtc gttccaggcc gggggctttg aagccctggt   1260

ggaaacagca ctgggtgccg ccgcacatcg ttcagccgag atggctgagc aactgggcaa   1320

atagtcgtcc cttaccaagg taatcaaaca tgctcggaat caatgacgct gccattttca   1380

tcatccagac cctgggcagc ctgtacctgc tgatcgtact gatgcgcttt atcctgcaac   1440

tggtgcgtgc gaacttctac aacccgctgt gccagttcgt ggtgaaggcc acccaaccgc   1500

tgctcaagcc gctgcgccgg gtgatcccga gcctgttcgg cctggacatg tcgtcgctgg   1560

tgctggcgct gttgctgcag attttgctgt tcgtggtgat cctgatgctc aatggatacc   1620

aggccttcac cgtgctgctg ttgccatggg gcctgatcgg gattttctcg ctgttcctga   1680

agatcatttt ctggtcgatg atcatcagcg tgatcctgtc ctgggtcgca ccgggtagcc   1740

gtagcccggg tgccgaattg gtggctcaga tcaccgagcc ggtgctggca cccttccgtc   1800

gcctgattcc gaacctgggt ggcctggata tctcgccgat cttcgcgttt atc          1853
```

<210> SEQ ID NO 7
<211> LENGTH: 272
<212> TYPE: PRT
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 7

```
Met Ser Asn Thr Arg Ile Ala Phe Ile Gly Ala Gly Asn Met Ala Ala
1               5                   10                  15

Ser Leu Ile Gly Gly Leu Arg Ala Lys Gly Leu Asp Ala Glu Gln Ile
            20                  25                  30

Arg Ala Ser Asp Pro Gly Ala Glu Thr Arg Glu Arg Val Arg Ala Glu
        35                  40                  45

His Gly Ile Gln Thr Phe Ala Asp Asn Ala Glu Ala Ile His Gly Val
    50                  55                  60

Asp Val Ile Val Leu Ala Val Lys Pro Gln Ala Met Lys Ala Val Cys
65                  70                  75                  80

Glu Ser Leu Ser Pro Ser Leu Gln Pro His Gln Leu Val Val Ser Ile
                85                  90                  95

Ala Ala Gly Ile Thr Cys Ala Ser Met Thr Asn Trp Leu Gly Ala Gln
            100                 105                 110

Pro Ile Val Arg Cys Met Pro Asn Thr Pro Ala Leu Leu Arg Gln Gly
        115                 120                 125

Val Ser Gly Leu Tyr Ala Thr Gly Glu Val Thr Ala Gln Gln Arg Asp
    130                 135                 140
```

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```
Gln Ala Gln Glu Leu Leu Ser Ala Val Gly Ile Ala Val Trp Leu Glu
145                 150                 155                 160

Gln Glu Gln Gln Leu Asp Ala Val Thr Ala Val Ser Gly Ser Gly Pro
                165                 170                 175

Ala Tyr Phe Phe Leu Leu Ile Glu Ala Met Thr Ala Ala Gly Val Lys
            180                 185                 190

Leu Gly Leu Pro His Asp Val Ala Glu Gln Leu Ala Glu Gln Thr Ala
        195                 200                 205

Leu Gly Ala Ala Lys Met Ala Val Gly Ser Glu Val Asp Ala Ala Glu
    210                 215                 220

Leu Arg Arg Arg Val Thr Ser Pro Gly Gly Thr Thr Gln Ala Ala Ile
225                 230                 235                 240

Glu Ser Phe Gln Ala Gly Gly Phe Glu Ala Leu Val Glu Thr Ala Leu
                245                 250                 255

Gly Ala Ala Ala His Arg Ser Ala Glu Met Ala Glu Gln Leu Gly Lys
            260                 265                 270
```

<210> SEQ ID NO 8
<211> LENGTH: 816
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas fluorescens

<400> SEQUENCE: 8

```
atgagcaaca cgcgtattgc ctttatcggc gccggtaaca tggcggccag cctgatcggt     60

ggcctgcggg ccaagggcct ggacgccgag cagatccgcg ccagcgaccc cggtgccgaa    120

acccgcgagc gcgtcagagc cgaacacggt atccagacct tcgccgataa cgccgaggcc    180

atccacggcg tcgatgtgat cgtgctggcg gtcaagcccc aggccatgaa ggccgtgtgc    240

gagagcctga gcccgagcct gcaaccccat caactggtgg tgtcgattgc cgctggcatc    300

acctgcgcca gcatgaccaa ctggctcggt gcccagccca ttgtgcgctg catgcccaac    360

accccggcgc tgctgcgcca gggcgtcagc ggtttgtatg ccactggcga agtcaccgcg    420

cagcaacgtg accaggccca ggaactgctg tctgcggtgg gcatcgccgt gtggctggag    480

caggaacagc aactggatgc ggtcaccgcc gtctccggca gcggcccggc ttacttcttc    540

ctgttgatcg aggccatgac ggccgcaggc gtcaagctgg gcctgcccca cgacgtggcc    600

gagcaactgg cggaacaaac cgccctgggc gccgccaaga tggcggtcgg cagcgaggtg    660

gatgccgccg aactgcgccg tcgcgtcacc tcgccaggtg gtaccacaca agcggctatt    720

gagtcgttcc aggccggggg ctttgaagcc ctggtggaaa cagcactggg tgccgccgca    780

catcgttcag ccgagatggc tgagcaactg ggcaaa                               816
```

<210> SEQ ID NO 9
<211> LENGTH: 1330
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 9

```
gacaccatcg aatggcgcaa aacctttcgc ggtatggcat gatagcgccc ggaagagagt     60

caattcaggg tggtgaatgt gaaaccagta acgttatacg atgtcgcaga gtatgccggt    120

gtctcttatc agaccgtttc ccgcgtggtg aaccaggcca gccacgtttc tgcgaaaacg    180

cgggaaaaag tggaagcggc gatggcggag ctgaattaca ttcccaaccg cgtggcacaa    240
```

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```
caactggcgg gcaaacagtc gttgctgatt ggcgttgcca cctccagtct ggccctgcac    300

gcgccgtcgc aaattgtcgc ggcgattaaa tctcgcgccg atcaactggg tgccagcgtg    360

gtggtgtcga tggtagaacg aagcggcgtc gaagcctgta aagcggcggt gcacaatctt    420

ctcgcgcaac gcgtcagtgg gctgatcatt aactatccgc tggatgacca ggatgccatt    480

gctgtggaag ctgcctgcac taatgttccg gcgttatttc ttgatgtctc tgaccagaca    540

cccatcaaca gtattatttt ctcccatgaa gacggtacgc gactgggcgt ggagcatctg    600

gtcgcattgg gtcaccagca aatcgcgctg ttagcgggcc cattaagttc tgtctcggcg    660

cgtctgcgtc tggctggctg gcataaatat ctcactcgca atcaaattca gccgatagcg    720

gaacgggaag gcgactggag tgccatgtcc ggttttcaac aaaccatgca aatgctgaat    780

gagggcatcg ttcccactgc gatgctggtt gccaacgatc agatggcgct gggcgcaatg    840

cgcgccatta ccgagtccgg gctgcgcgtt ggtgcggata tctcggtagt gggatacgac    900

gataccgaag acagctcatg ttatatcccg ccgtcaacca ccatcaaaca ggattttcgc    960

ctgctggggc aaaccagcgt ggaccgcttg ctgcaactct ctcagggcca ggcggtgaag   1020

ggcaatcagc tgttgcccgt ctcactggtg aaaagaaaaa ccaccctggc gcccaatacg   1080

caaaccgcct ctccccgcgc gttggccgat tcattaatgc agctggcacg acaggtttcc   1140

cgactggaaa gcgggcagtg agcgcaacgc aattaatgtg agttagctca ctcattaggc   1200

accccaggct ttacacttta tgcttccggc tcgtatgttg tgtggaattg tgagcggata   1260

acaatttcac acaggaaaca gctatgacca tgattacgga ttcactggcc gtcgttttac   1320

aacgtcgtga                                                          1330
```

<210> SEQ ID NO 10
<211> LENGTH: 359
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 10

```
Met Lys Pro Val Thr Leu Tyr Asp Val Ala Glu Tyr Ala Gly Val Ser
1               5                   10                  15

Tyr Gln Thr Val Ser Arg Val Val Asn Gln Ala Ser His Val Ser Ala
            20                  25                  30

Lys Thr Arg Glu Lys Val Glu Ala Ala Met Ala Glu Leu Asn Tyr Ile
        35                  40                  45

Pro Asn Arg Val Ala Gln Gln Leu Ala Gly Lys Gln Ser Leu Leu Ile
    50                  55                  60

Gly Val Ala Thr Ser Ser Leu Ala Leu His Ala Pro Ser Gln Ile Val
65                  70                  75                  80

Ala Ala Ile Lys Ser Arg Ala Asp Gln Leu Gly Ala Ser Val Val Val
                85                  90                  95

Ser Met Val Glu Arg Ser Gly Val Glu Ala Cys Lys Ala Ala Val His
            100                 105                 110

Asn Leu Leu Ala Gln Arg Val Ser Gly Leu Ile Ile Asn Tyr Pro Leu
        115                 120                 125

Asp Asp Gln Asp Ala Ile Ala Val Glu Ala Ala Cys Thr Asn Val Pro
    130                 135                 140

Ala Leu Phe Leu Asp Val Ser Asp Gln Thr Pro Ile Asn Ser Ile Phe
145                 150                 155                 160
```

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```
Ser His Glu Asp Gly Thr Arg Leu Gly Val Glu His Leu Val Ala Leu
                165                 170                 175

Gly His Gln Gln Ile Ala Leu Leu Ala Gly Pro Leu Ser Ser Val Ser
            180                 185                 190

Ala Arg Leu Arg Leu Ala Gly Trp His Lys Tyr Leu Thr Arg Asn Gln
        195                 200                 205

Ile Gln Pro Ile Ala Glu Arg Glu Gly Asp Trp Ser Ala Met Ser Gly
    210                 215                 220

Phe Gln Gln Thr Met Gln Met Leu Asn Glu Gly Ile Val Pro Thr Ala
225                 230                 235                 240

Met Leu Val Ala Asn Asp Gln Met Ala Leu Gly Ala Met Arg Ala Ile
                245                 250                 255

Thr Glu Ser Gly Leu Arg Val Gly Ala Asp Ile Ser Val Val Gly Tyr
            260                 265                 270

Asp Asp Thr Glu Asp Ser Ser Cys Tyr Ile Pro Pro Ser Thr Thr Ile
        275                 280                 285

Lys Gln Asp Phe Arg Leu Leu Gly Gln Thr Ser Val Asp Arg Leu Leu
    290                 295                 300

Gln Leu Ser Gln Gly Gln Ala Val Lys Gly Asn Gln Leu Leu Pro Val
305                 310                 315                 320

Ser Leu Val Lys Arg Lys Thr Thr Leu Ala Pro Asn Thr Gln Thr Ala
                325                 330                 335

Ser Pro Arg Ala Leu Ala Asp Ser Leu Met Gln Leu Ala Arg Gln Val
            340                 345                 350

Ser Arg Leu Glu Ser Gly Gln
        355
```

<210> SEQ ID NO 11
<211> LENGTH: 1320
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 11

```
gacaccatcg aatggtgcaa aacctttcgc ggtatggcat gatagcgccc ggaagagagt     60

caattcaggg tggtgaatgt gaaaccagta acgttatacg atgtcgcaga gtatgccggt    120

gtctcttatc agaccgtttc ccgcgtggtg aaccaggcca gccacgtttc tgcgaaaacg    180

cgggaaaaag tggaagcggc gatggcggag ctgaattaca ttcccaaccg cgtggcacaa    240

caactggcgg gcaaacagtc gttgctgatt ggcgttgcca cctccagtct ggccctgcac    300

gcgccgtcgc aaattgtcgc ggcgattaaa tctcgcgccg atcaactggg tgccagcgtg    360

gtggtgtcga tggtagaacg aagcggcgtc gaagcctgta aagcggcggt gcacaatctt    420

ctcgcgcaac gcgtcagtgg gctgatcatt aactatccgc tggatgacca ggatgccatt    480

gctgtggaag ctgcctgcac taatgttccg gcgttatttc ttgatgtctc tgaccagaca    540

cccatcaaca gtattatttt ctcccatgaa gacggtacgc gactgggcgt ggagcatctg    600

gtcgcattgg gtcaccagca aatcgcgctg ttagcgggcc cattaagttc tgtctcggcg    660

cgtctgcgtc tggctggctg gcataaatat ctcactcgca atcaaattca gccgatagcg    720

gaacgggaag gcgactggag tgccatgtcc ggttttcaac aaaccatgca aatgctgaat    780

gagggcatcg ttcccactgc gatgctggtt gccaacgatc agatggcgct gggcgcaatg    840
```

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cgcgccatta ccgagtccgg gctgcgcgtt ggtgcggata tctcggtagt gggatacgac 900

gataccgaag acagctcatg ttatatcccg ccgtcaacca ccatcaaaca ggattttcgc 960

ctgctggggc aaaccagcgt ggaccgcttg ctgcaactct ctcagggcca ggcggtgaag 1020

ggcaatcagc tgttgcccgt ctcactggtg aaaagaaaaa ccaccctggc gcccaatacg 1080

caaaccgcct ctccccgcgc gttggccgat tcattaatgc agctggcacg acaggtttcc 1140

cgactggaaa gcgggcagtg agcgcaacgc aattaatgtg agttagctca ctcattaggc 1200

accccaggct ttacacttta tgcttccggc tcgtatgttg tgtggaattg tgagcggata 1260

acaatttcac acaggaaaca gctatgacca tgattacgga ttcactggcc gtcgttttac 1320

<210> SEQ ID NO 12
<211> LENGTH: 1324
<212> TYPE: DNA
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 12

agcggcatgc atttacgttg acaccacctt tcgcggtatg gcatgatagc gcccggaaga 60

gagtcaattc agggtggtga atgtgaaacc agtaacgtta tacgatgtcg cagagtatgc 120

cggtgtctct tatcagaccg tttcccgcgt ggtgaaccag gccagccacg tttctgcgaa 180

aacgcgggaa aaagtggaag cggcgatggc ggagctgaat tacattccca accgcgtggc 240

acaacaactg gcgggcaaac agtcgttgct gattggcgtt gccacctcca gtctggccct 300

gcacgcgccg tcgcaaattg tcgcggcgat taaatctcgc gccgatcaac tgggtgccag 360

cgtggtggtg tcgatggtag aacgaagcgg cgtcgaagcc tgtaaagcgg cggtgcacaa 420

tcttctcgcg caacgcgtca gtgggctgat cattaactat ccgctggatg accaggatgc 480

cattgctgtg gaagctgcct gcactaatgt tccggcgtta tttcttgatg tctctgacca 540

gacacccatc aacagtatta ttttctccca tgaagacggt acgcgactgg gcgtggagca 600

tctggtcgca ttgggtcacc agcaaatcgc gctgttagcg ggcccattaa gttctgtctc 660

ggcgcgtctg cgtctggctg gctggcataa atatctcact cgcaatcaaa ttcagccgat 720

agcggaacgg gaaggcgact ggagtgccat gtccggtttt caacaaacca tgcaaatgct 780

gaatgagggc atcgttccca ctgcgatgct ggttgccaac gatcagatgg cgctgggcgc 840

aatgcgcgcc attaccgagt ccgggctgcg cgttggtgcg gatatctcgg tagtgggata 900

cgacgatacc gaagacagct catgttatat cccgccgtca accaccatca aacaggattt 960

tcgcctgctg gggcaaacca gcgtggaccg cttgctgcaa ctctctcagg gccaggcggt 1020

gaagggcaat cagctgttgc ccgtctcact ggtgaaaaga aaaaccaccc tggcgcccaa 1080

tacgcaaacc gcctctcccc gcgcgttggc cgattcatta atgcagctgg cacgacaggt 1140

ttcccgactg gaaagcgggc agtgagcgca acgcaattaa tgtgagttag ctcactcatt 1200

aggcacccca ggctttacac tttatgcttc cggctcgtat gttgtgtgga attgtgagcg 1260

gataacaatt tcacacagga aacagctatg accatgatta cggattcact ggccgtcgtt 1320

ttac 1324

<210> SEQ ID NO 13
<211> LENGTH: 3001
<212> TYPE: DNA
<213> ORGANISM: Pseudomonas fluorescens

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<400> SEQUENCE: 13

ctacccagaa cgaagatcag cgcctcaatg gcctcaaggt tctactggtc gatgattcag 60

ccgaagtcgt tgaggtgctg aacatgctgc tggaaatgga aggcgcccaa gtgagcgcct 120

tcagcgaccc tttgagcgcg cttgaaacag cccgggatgc ccattacgac gtgattattt 180

cggacatcgg catgccgaaa atgaatggcc atgagctgat gcagaagctg cgtaaagtag 240

gccaccttcg acaggctccc gccatcgcct taacgggcta tggcgctggc aatgaccaga 300

aaaaggcgac tgaatcgggc tttaatgcgc atgtcagcaa acccgttggc catgattcgc 360

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We claim:

1) An auxotrophic Pseudomonad cell for use in a bacterial expression system that comprises a nucleic acid construct comprising:

a. a nucleic acid encoding a recombinant polypeptide; and,

b. a nucleic acid encoding at least one polypeptide that restores prototrophy to the auxotrophic host cell.

2) The cell of claim 1, wherein the Pseudomonad is *Pseudomonas fluorescens.*

3) The cell of claim 1, wherein the cell is auxotrophic for uracil.

4) The cell of claim 1, wherein the cell is auxotrophic for proline.

5) The cell of claim 1, wherein the auxotrophic cell is auxotrophic for more than one metabolite.

6) The cell of claim 5, wherein the cell is auxotrophic for uracil and proline.

7) The cell of claim 1, wherein the prototrophy restoring polypeptide is an enzyme active in the biosynthesis of a metabolite required for cell survival.

8) The cell of claim 7, wherein the enzyme is orotodine-5'-phosphate decarboxylase.

9) The cell of claim 8, wherein the enzyme is encoded by the nucleic acid sequence selected from the group consisting of SEQ. ID. 1 and 3.

10) The cell of claim 7, wherein the enzyme comprises the amino acid sequence of SEQ ID No. 2.

11) The cell of claim 7, wherein the enzyme is Δ^1-pyrroline-5-carboxylate reductase.

12) The cell of claim 11, wherein the enzyme is encoded by the nucleic acid sequence selected from the group consisting of SEQ. ID. NO. 6 and 8.

13) The cell of claim 11, wherein the enzyme comprises the amino acid sequence of SEQ. ID. No. 7.

14) The cell of claim 1, wherein the auxotrophic cell is produced by disabling a pyrF gene.

15) The cell of claim 14, wherein the disabled pyrF gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 1 and SEQ. ID. No. 3.

16) The cell of claim 1, wherein the auxotrophic cell is produced by disabling a proC gene.

17) The cell of claim 16, wherein the disabled proC gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 6 and SEQ. ID. No. 8.

18) The cell of claim 1, wherein the auxotrophic cell is produced by disabling a pyrF gene and a proC gene.

19) The cell of claim 18, wherein the disabled pyrF gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 1 and SEQ. ID. No.3, and the disabled proC gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 6 and SEQ. ID. NO. 9.

20) The cell of claim 1, wherein the cell also contains a chromosomal lacI insert that is other than as part of a PlacI-lacI-lacZYA operon.

21) The cell of claim 20, wherein the lacI gene is selected from the group consisting of lacI, $lacI^{Q}$ and $lacI^{Q1}$.

22) The cell of claim 1, wherein the nucleic acid construct further comprises at least one lacOid sequence.

23) The cell of claim 22, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

24) The cell of claim 1, wherein the nucleic acid construct further comprising more than one lac operator sequences.

25) The cell of claim 24, wherein at least one lac operator sequence is located 5' of a promoter, and at least one lac operator sequence is located 3' of a promoter.

26) The cell of claim 25, wherein at least one lac operator sequence is a lacOid sequence.

27) The cell of claim 26, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

28) A genetically modified Pseudomonad cell for use in a bacterial expression system, wherein the modification comprises at least one chromosomal insertion of a lacI gene, wherein the lacI gene is other than as part of a whole or truncated PlacI-lacI-lacZYA operon.

29) The cell of claim 28, wherein the Pseudomonad is *Pseudomonas fluorescens.*

30) The cell of claim 28, wherein the lacI gene is selected from the group consisting of lacI, $lacI^{Q}$ and $lacI^{Q1}$.

31) The cell of claim 28, wherein the lacI gene is inserted in the levansucrase locus.

32) The cell of claim 28, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

33) The cell of claim 32, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

34) The cell of claim 32, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

35) The cell of claim 28, further comprising a nucleic acid comprising at least one lacOid sequence.

36) The cell of claim 35, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

37) The cell of claim 28 further comprising a nucleic acid comprising more than one lac operator sequence.

38) The cell of claim 37, wherein at least one lac operator sequence is a lacOid sequence.

39) The cell of claim 38, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

40) A Pseudomonad cell for use in a bacterial expression system comprising a nucleic acid construct comprising at least one lacOid operator sequence.

41) The cell of claim 40, wherein the Pseudomonad is a *Pseudomonas fluorescens.*

42) The cell of claim 40, wherein the lacOid sequence is located 3' of a promoter.

43) The cell of claim 40, wherein the lacOid sequence is located 5' of a promoter.

44) The cell of claim 40, wherein lacOid sequences are located 3' and 5' of a promoter.

45) The cell of claim 40, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

46) The cell of claim 45, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

47) The cell of claim 45, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

48) The cell of claim 40, wherein the cell contains a chromosomal insertion of a lacI gene, wherein the lacI gene is other than as part of a whole or truncated Plac-lacI-lacZYA operon.

49) The cell of claim 40, wherein the lacOid sequence is selected from the group consisting of SEQ ID NO. 14 and SEQ. ID. NO. 59.

50) A Pseudomonad cell for use in a bacterial expression system comprising a nucleic acid construct comprising more than one lac operator sequence.

51) The cell of claim 50, wherein at least one lac operator is a lacOid sequence.

52) The cell of claim 51, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

53) The cell of claim 51, wherein the lacOid sequence is 5' or 3' of the promoter.

54) The cell of claim 51 wherein the lacOid sequence is 5' and 3' of the promoter.

55) The cell of claim 50, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

56) The cell of claim 55, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

57) The cell of claim 55, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

58) The cell of claim 50, wherein the cell contains a chromosomal insertion of a lacI gene, wherein the lacI gene is other than as part of a whole or truncated Plac-lacI-lacZYA operon.

59) The cell of claim 50, wherein the Pseudomonad is *Pseudomonas fluorescens*.

60) A process for producing a recombinant polypeptide comprising:

a. expressing a nucleic acid encoding the recombinant polypeptide in a Pseudomonad cell that has been genetically modified to create an auxotrophy for at least one metabolite;

b. expressing a nucleic acid encoding a polypeptide that restores prototrophy to the auxotrophic cell; and,

c. growing the cell on a medium that lacks the auxotrophic metabolite.

61) The process of claim 60, wherein the Pseudomonad is *Pseudomonas fluorescens*.

62) The process of claim 60, wherein the cell is auxotrophic for uracil.

63) The process of claim 60, wherein the cell is auxotrophic for proline.

64) The process of claim 60, wherein the auxotrophic cell is auxotrophic for more than one metabolite.

65) The process of claim 64, wherein the cell is auxotrophic for uracil and proline.

66) The process of claim 60, wherein the prototrophy restoring polypeptide is an enzyme active in the biosynthesis of a metabolite required for cell survival.

67) The process of claim 66, wherein the enzyme is orotodine-5'-phosphate decarboxylase.

68) The process of claim 67, wherein the enzyme is encoded by the nucleic acid sequence selected from the group consisting of SEQ. ID. 1 and 3.

69) The process of claim 68, wherein the enzyme comprises the amino acid sequence of SEQ ID No. 2.

70) The process of claim 66, wherein the enzyme is Δ^1-pyrroline-5-carboxylate reductase.

71) The process of claim 70, wherein the enzyme is encoded by the nucleic acid sequence selected from the group consisting of SEQ. ID. NO. 6 and 8.

72) The process of claim 70, wherein the enzyme comprises the amino acid sequence of SEQ. ID. No. 7.

73) The process of claim 60, wherein the auxotrophic cell is produced by disabling a pyrF gene.

74) The process of claim 73, wherein the disabled pyrF gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 1 and SEQ. ID. No. 3.

75) The process of claim 60, wherein the auxotrophic cell is produced by disabling a proC gene.

76) The process of claim 75, wherein the disabled proC gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 6 and SEQ. ID. No. 8.

77) The process of claim 60, wherein the auxotrophic cell is produced by disabling a pyrF gene and a proC gene.

78) The process of claim 77, wherein the disabled pyrF gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 1 and SEQ. ID. No. 3, and the disabled proC gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 6 and SEQ. ID. NO. 9.

79) The process of claim 60, wherein the cell also contains a chromosomal lacI insert that is other than as part of a PlacI-lacI-lacZYA operon.

80) The process of claim 79, wherein the lacI gene is selected from the group consisting of lacI, $lacI^{Q,}$ and lac^{Q1}.

81) The process of claim 60, wherein the nucleic acid encoding the recombinant polypeptide further comprises at least one lacOid sequence.

82) The proves of claim 81, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

83) The process of claim 60, wherein the nucleic acid encoding the recombinant polypeptide further comprises more than one lac operator sequences.

84) The process of claim 83, wherein at least one lac operator sequence is located 5' of a promoter, and at least one lac operator sequence is located 3' of a promoter.

85) The process of claim 84, wherein at least one lac operator sequence is a lacOid sequence.

86) The process of claim 85, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

87) A process for producing a recombinant polypeptide comprising expressing a nucleic acid encoding the recombinant polypeptide in a Pseudomonad that comprises at least one chromosomal insertion of a lacI gene, wherein the lacI gene is other than as part of a whole or truncated PlacI-lacI-lacZYA operon.

88) The process of claim 87, wherein the Pseudomonad is *Pseudomonas fluorescens*.

89) The process of claim 87, wherein the lacI gene is selected from the group consisting of lacI, $lacI^{Q,}$ and $lacI^{Q1}$.

90) The process of claim 87, wherein the lacI gene is inserted in the levansucrase locus.

91) The process of claim 87, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

92) The process of claim 91, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

93) The process of claim 91, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

94) The process of claim 87, wherein the nucleic acid encoding the recombinant polypeptide comprises at least one lacOid sequence.

95) The process of claim 94, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

96) The process of claim 87, wherein the nucleic acid encoding the recombinant polypeptide comprises more than one lac operator sequence.

97) The process of claim 96, wherein at least one lac operator sequence is a lacOid sequence.

98) The process of claim 97, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

99) A process for producing a recombinant polypeptide comprising expressing a nucleic acid encoding the recombinant polypeptide in a Pseudomonad cell, wherein the nucleic acid further comprises at least one lac operator sequence, wherein the lac operator sequence is a lacOid sequence.

100) The process of claim 99, wherein the Pseudomonad is a *Pseudomonas fluorescens*.

101) The process of claim 99, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

102) The process of claim 99, wherein at least one lacOid sequence is located 3' of a promoter.

103) The process of claim 99, wherein at least one lacOid sequence is located 5' of a promoter.

104) The process of claim 99, wherein at least one lacOid sequence is located 3' and 5' of a promoter.

105) The process of claim 99, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

106) The process of claim 105, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

107) The process of claim 105, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

108) The process of claim 99, wherein the cell contains a chromosomal insertion of a lacI gene, wherein the lacI gene is other than as part of a whole or truncated Plac-lacI-lacZYA operon.

109) A process for producing a recombinant polypeptide comprising expressing a nucleic acid encoding the recombinant polypeptide in a Pseudomonad cell, wherein the nucleic acid further comprises more than one lac operator sequence.

110) The process of claim 109, wherein at least one lac operator is a lacOid sequence.

111) The process of claim 110, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

112) The process of claim 110, wherein the lacOid sequence is 5' or 3' of the promoter.

113) The process of claim 110, wherein the lacOid sequence is 5' and 3' of the promoter.

114) The process of claim 109, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

115) The process of claim 114, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

116) The process of claim 114, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

117) The process of claim 109, wherein the cell contains a chromosomal insertion of a lacI gene, wherein the lacI gene is other than as part of a whole or truncated Plac-lacI-lacZYA operon.

118) The process of claim 109, wherein the Pseudomonad is *Pseudomonas fluorescens*

119) A process for modulating the expression of a recombinant polypeptide in a host cell comprising:

a. selecting a Pseudomonad cell, wherein the cell has been genetically modified by chromosomally inserting a lacI gene into the cell, wherein the lacI gene is other than as part of a whole or truncated PlacI-lacI-lacZYA operon; and,

b. introducing into the cell a nucleic acid construct comprising a LacI protein promoter operably attached to a nucleic acid encoding the recombinant polypeptide.

120) The process of claim 119, wherein the Pseudomonad is *Pseudomonas fluorescens.*

121) The process of claim 119, wherein the lacI gene is selected from the group consisting of lacI, $lacI^{Q,}$ and $lacI^{Q1}$.

122) The process of claim 119, wherein the lacI gene is inserted in the levansucrase locus.

123) The process of claim 119, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

124) The process of claim 123, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

125) The process of claim 123, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

126) The process of claim 119, wherein the nucleic acid encoding the recombinant polypeptide comprises at least one lacOid sequence.

127) The process of claim 126, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

128) The process of claim 126, wherein at least one lacOid sequence is located 3' of the promoter.

129) The process of claim 126, wherein at least one lacOid sequence is located 5' of the promoter.

130) The process of claim 126, wherein lacOid sequence are located 5' and 3' of the promoter.

131) The process of claim 119, wherein the nucleic acid encoding the recombinant polypeptide comprises more than one lac operator sequence.

132) The process of claim 131, wherein at least one lac operator sequence is a lacOid sequence.

133) The process of claim 132, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

134) A process for modulating the expression of a recombinant polypeptide in a host cell comprising:

a. selecting a Pseudomonad cell; and

b. introducing a nucleic acid construct comprising:

i. a nucleic acid encoding the recombinant polypeptide, and,

ii. at least one lacOid operator sequence.

135) The process of claim 134, wherein the Pseudomonad is a *Pseudomonas fluorescens.*

136) The process of claim 134, wherein the lacOid sequence is located 3' of a promoter.

137) The process of claim 134, wherein the lacOid sequence is located 5' of a promoter.

138) The process of claim 134, wherein lacOid sequences are located 3' and 5' of a promoter.

139) The process of claim 134, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

140) The process of claim 139, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

141) The process of claim 139, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

142) The process of claim 134, wherein the cell contains a chromosomal insertion of a lacI gene, wherein the lacI gene is other than as part of a whole or truncated Plac-lacI-lacZYA operon.

143) The process of claim 134, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO. 14 and SEQ. ID. NO. 59.

144) A process for modulating the expression of a recombinant polypeptide in a host cell comprising:

a. selecting a Pseudomonad cell; and

b. introducing a nucleic acid construct comprising:

i. a nucleic acid encoding the recombinant polypeptide, and,

ii. more than one lac operator sequence.

145) The process of claim 144, wherein at least one lac operator is a lacOid sequence.

146) The process of claim 145, wherein the lacOid sequence is selected from the group consisting of SEQ. ID. NO.14 and SEQ. ID. NO. 59.

147) The process of claim 145, wherein the lacOid sequence is 5' or 3' of the promoter.

148) The process of claim 145 wherein the lacOid sequence is 5' and 3' of the promoter.

149) The process of claim 144, wherein the cell has been further modified to create an auxotrophy for at least one metabolite in the cell.

150) The process of claim 149, wherein the auxotrophy is created by modification to a gene selected from the group consisting of pyrF and proC.

151) The process of claim 149, wherein the auxotrophy is created by modification to both the pyrF and the proC gene.

152) The process of claim 144, wherein the cell contains a chromosomal insertion of a lacI gene, wherein the lacI gene is other than as part of a whole or truncated Plac-lacI-lacZYA operon.

153) The process of claim 144, wherein the Pseudomonad is *Pseudomonas fluorescens.*

154) A process for the production of a recombinant polypeptide in the absence of antibiotics comprising:

a. selecting a Pseudomonad cell, wherein the cell has been genetically modified to induce an auxotrophy for at least one metabolite, thereby creating an auxotrophic cell;

b. introducing into the cell a nucleic acid construct comprising

i. a nucleic acid encoding the recombinant polypeptide; and

ii. a nucleic acid encoding a polypeptide that restores prototrophy to the auxotrophic host cell;

c. expressing the recombinant polypeptide and prototrophy restoring polypeptide in the cell; and,

d. growing the cell on a medium that lacks the auxotrophic metabolite.

155) The process of claim 154, wherein the Pseudomonad is *Pseudomonas fluorescens.*

156) The process of claim 154, wherein the cell is auxotrophic for uracil.

157) The process of claim 154, wherein the cell is auxotrophic for proline.

158) The process of claim 154, wherein the auxotrophic cell is auxotrophic for more than one metabolite.

159) The process of claim 158, wherein the cell is auxotrophic for uracil and proline.

160) The process of claim 154, wherein the prototrophy restoring polypeptide is an enzyme active in the biosynthesis of a metabolite required for cell survival.

161) The process of claim 160, wherein the enzyme is orotodine-5'-phosphate decarboxylase.

162) The process of claim 161, wherein the enzyme is encoded by the nucleic acid sequence selected from the group consisting of SEQ. ID. 1 and 3.

163) The process of claim 160, wherein the enzyme comprises the amino acid sequence of SEQ ID No. 2.

164) The process of claim 160, wherein the enzyme is Δ^1-pyrroline-5-carboxylate reductase.

165) The process of claim 164, wherein the enzyme is encoded by the nucleic acid sequence selected from the group consisting of SEQ. ID. NO. 6 and 8.

166) The process of claim 164, wherein the enzyme comprises the amino acid sequence of SEQ. ID. No. 7.

167) The process of claim 154, wherein the auxotrophic cell is produced by disabling a pyrF gene.

168) The process of claim 167, wherein the disabled pyrF gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No.1 and SEQ. ID. No. 3.

169) The process of claim 154, wherein the auxotrophic cell is produced by disabling a proC gene.

170) The process of claim 169, wherein the disabled proC gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 6 and SEQ. ID. No. 8.

171) The process of claim 154, wherein the auxotrophic cell is produced by disabling a pyrF gene and a proC gene.

172) The process of claim 171, wherein the disabled pyrF gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No.1 and SEQ. ID. No. 3, and the disabled proC gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 6 and SEQ. ID. NO.9.

173) The process of claim 154, wherein the cell also contains a chromosomal lacI insert that is other than as part of a PlacI-lacI-lacZYA operon.

174) The process of claim 173, wherein the lacI gene is selected from the group consisting of lacI, $lacI^{Q}$, and $lacI^{Q1}$.

175) The process of claim 154, wherein the nucleic acid construct further comprises at least one lacOid sequence.

176) The process of claim 154, wherein the nucleic acid construct further comprising more than one lac operator sequences.

177) The process of claim 176, wherein at least one lac operator sequence is located 5' of a promoter, and at least one lac operator sequence is located 3' of a promoter.

178) The process of claim 177, wherein at least one lac operator sequence is a lacOid sequence.

179) A process for the production of a recombinant polypeptide in the absence of antibiotics wherein cross feeding inhibition is minimized during selection comprising:

a. selecting a Pseudomonad cell, wherein the cell has been genetically modified to induce an auxotrophy for at least one metabolite, thereby creating an auxotrophic cell;

b. introducing into the cell a nucleic acid construct comprising

i. a nucleic acid encoding a recombinant polypeptide; and

ii. a nucleic acid encoding a polypeptide that restores prototrophy to the auxotrophic host cell;

c. expressing the recombinant polypeptide and the prototrophy restoring polypeptide in the cell; and,

d. growing the cell on a medium that lacks the auxotrophic metabolite.

180) The process of claim 179, wherein the Pseudomonad is *Pseudomonas fluorescens.*

181) The process of claim 179, wherein the cell is auxotrophic for uracil.

182) The process of claim 179, wherein the cell is auxotrophic for proline.

183) The process of claim 179, wherein the auxotrophic cell is auxotrophic for more than one metabolite.

184) The process of claim 183, wherein the cell is auxotrophic for uracil and proline.

185) The process of claim 179, wherein the prototrophy restoring polypeptide is an enzyme active in the biosynthesis of a metabolite required for cell survival.

186) The process of claim 185, wherein the enzyme is orotodine-5'-phosphate decarboxylase.

187) The process of claim 186, wherein the enzyme is encoded by the nucleic acid sequence selected from the group consisting of SEQ. ID. 1 and 3.

188) The process of claim 185, wherein the enzyme comprises the amino acid sequence of SEQ ID No. 2.

189) The process of claim 185, wherein the enzyme is Δ^1-pyrroline-5-carboxylate reductase.

190) The process of claim 189, wherein the enzyme is encoded by the nucleic acid sequence selected from the group consisting of SEQ. ID. NO. 6 and 8.

191) The process of claim 189, wherein the enzyme comprises the amino acid sequence of SEQ. ID. No. 7.

192) The process of claim 179, wherein the auxotrophic cell is produced by disabling a pyrF gene.

193) The process of claim 192, wherein the disabled pyrF gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 1 and SEQ. ID. No. 3.

194) The process of claim 179, wherein the auxotrophic cell is produced by disabling a proC gene.

195) The process of claim 194, wherein the disabled proC gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 6 and SEQ. ID. No. 8.

196) The process of claim 179, wherein the auxotrophic cell is produced by disabling a pyrF gene and a proC gene.

197) The process of claim 196, wherein the disabled pyrF gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 1 and SEQ. ID. No.3, and the disabled proC gene comprises the nucleic acid selected from the group consisting of SEQ. ID. No. 6 and SEQ. ID. NO. 9.

198) The process of claim 179, wherein the cell also contains a chromosomal lacI insert that is other than as part of a PlacI-lacI-lacZYA operon.

199) The process of claim 198, wherein the lacI gene is selected from the group consisting of lacI, $lacI^{Q}$, and $lacI^{Q1}$.

200) The process of claim 199, wherein the nucleic acid construct further comprises at least one lacOid sequence.

201) The process of claim 179, wherein the nucleic acid construct further comprising more than one lac operator sequences.

202) The process of claim 201, wherein at least one lac operator sequence is located 5' of a promoter, and at least one lac operator sequence is located 3' of a promoter.

203) The process of claim 202, wherein at least one lac operator sequence is a lacOid sequence.

204) A *Pseudomonas fluorescens* pyrF gene, or nucleic acid that hybridizes with the pyrF gene, comprising the nucleic acid sequence selected from the group consisting of SEQ. ID. No. 1 or 3.

205) The gene of claim 204, wherein the nucleic acid comprises the sequence of SEQ. ID. No. 1.

206) The gene of claim 204, wherein the nucleic acid comprises the sequence of SEQ. ID. No. 3

207) A *Pseudomonas fluorescens* proC gene, or nucleic acid that hybridizes with the proC gene, comprising the nucleic acid sequence selected from the group consisting of SEQ. ID. No. 6 or 8.

208) The gene of claim 207, wherein the nucleic acid comprises the sequence of SEQ ID No. 6.

209) The gene of claim 207, wherein the nucleic acid comprises the sequence of SEQ ID No. 8.

210) A nucleic acid construct comprising:

a. a nucleic acid encoding a recombinant polypeptide; and

b. a nucleic acid encoding a pyrF gene isolated from a *Pseudomonas fluorescens.*

211) The construct of claim 210, wherein the pyrF gene comprises the nucleic acid sequence of SEQ. ID No. 1.

212) The construct of claim 210, wherein the pyrF gene comprises the nucleic acid sequence of SEQ. ID No. 3.

213) A nucleic acid construct comprising:

a. a nucleic acid encoding a recombinant polypeptide; and

b. a nucleic acid encoding a proC gene isolated from a *Pseudomonas fluorescens.*

214) The construct of claim 213, wherein the proC gene comprises the nucleic acid sequence of SEQ ID No. 6.

215) The construct of claim 213, wherein the proC gene comprises the nucleic acid sequence of SEQ ID No. 8.

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